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JC17 Rec'd PCT/PTO 31 MAY 2001

FORM PTO-1390(modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE
(REV 10-95)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C., 371	ATTORNEY'S DOCKET NUMBER **01/22115**
	U.S. APPLICATION NO. (IF KNOWN), SEE 37 CFR **09/857069**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IL99/00651	01 December 1999	01 December 1998

TITLE OF INVENTION

Methods For Bulk Stable Introduction And Expression Of Foreign Genes Into Eukaryotic Parasites

APPLICANT(S) FOR DO/EO/US

Joseph HAMBURGER and Avraham LABAN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO.US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(3)(2)
 a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 b. ☐ has been transmitted by the International Bureau.
 c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(3)(2).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)
 a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 b. ☒ have been transmitted by the International Bureau.
 c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19(35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)5)).

Items 13 to 18 below concern document(s) of information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A FIRST preliminary amendment.
 A SECOND or SUBSEQUENT preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

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U.S. APPLICATION NO. (IF KNOWN), See 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/857069	PCT/IL99/00651	01/22115

20. The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a) (1) – (5)):		
☐ Search Report has been prepared by the EPO or JPO $ 860		
☒ International preliminary examination fee paid to USPTO (37 CFR 1,482) $ 690		
☐ No international preliminary examination fee paid to USPTO (37 CFR 1,482) but international search fee paid to USPTO (37 CFR 1,445(a)(2)) $ 710		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $ 1000		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $ 100		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$690.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e))	$.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =		x $ 18	$.00	
Independent claims	1 - 3 =		x $ 80	$.00	
Multiple Dependent Claims (**check if applicable**)			☐	$.00	
TOTAL OF ABOVE CALCULATIONS =				$690.00	
Reduction of ½ for filing by small entity, if applicable. ☒				$345.00	
SUBTOTAL =				$345.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f) +				$.00	
TOTAL NATIONAL FEE =				$345.00	
Fee for recording the enclosed assignment (37 CFR 1.2(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable) ☒				$40.00	
TOTAL FEES ENCLOSED =				$385.00	
				Amount to be refunded:	$
				charged	$

☐ A check in the amount of $ _____ to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **50-1407** in the amount of $ 385.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-1407**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGISTRATION NUMBER

May 21, 2001
DATE

09/857069
JC18 Rec'd PCT/PTO 31 MAY 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Applicant:	§	
	§	
Joseph HAMBURGER et al	§	
	§	
Serial No.: 09/	§	
	§	
Filed: Concurrently	§	Group Art Unit:
	§	
For: Methods for Bulk Stable Introduction and Expression Of Foreign Genes Into Eukaryotic Parasites	§	Attorney Docket: 00/22115
	§	
	§	
	§	

Director of the Patent and Trademark Office
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to examining the National phase application of PCT/IL99/00651, please amend the claims in the manner as submitted in response to the Written Opinion:

In the claims:

Cancel all the claims as recited in the published WO 00/32804 and substitute therefor new claims 1-17:

1. A method of genetically modifying a eukaryotic diploid parasite, the method comprising the step of directly transforming a multicellular differentiated developmental stage of the eukaryotic diploid parasite using a group transformation method, so as to obtain a genetically modified eukaryotic diploid parasite.

2. The method of claim 1, wherein the parasite is a worm.

3. The method of claim 2, wherein said worm is a flat worm.

4. The method of claim 3, wherein said flat worm is a trematode.

5. The method of claim 4, wherein said trematode is a schistosome.

6. The method of claim 5, wherein said developmental stage is miracidia, either within ova or after hatching.

7. The method of claim 1, wherein the parasite is infective to human or animal.

8. The method of claim 1, wherein the parasite is infective to human.

9. The method of claim 5, wherein said schistosome is infective to human.

10. The method of claim 5, wherein said schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis.*

11. The method of claim 1, wherein the parasite is sensitive to a known drug.

12. The method of claim 1, wherein said group transformation method is selected from the group consisting of electroporation, chemical transformation, lipofection and biolistic bombardment.

13. The method of claim 1, wherein said transgene is integrated in the parasite genome.

14. The method of claim 1, wherein said transgene is integrated in a selected genomic locus in the parasite genome.

15. The method of claim 14, wherein said selected genomic locus is a repetitive sequence.

16. The method of claim 14, wherein said selected genomic locus is a unique sequence.

17. The method of claim 1, wherein the parasite has distinguishable sexes.

REMARKS

The claims as presented hereinabove now correspond to those in the PCT application after the Written Opinion.

Respectfully submitted,

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Date: May 29, 2001

METHODS FOR BULK STABLE INTRODUCTION AND EXPRESSION OF FOREIGN GENES INTO EUKARYOTIC PARASITES

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to transgenic eukaryotic parasites such as parasitic worms and, more particularly, to the use of transgenic eukaryotic parasites, such as parasitic worms as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals. Most particularly, the present invention relates to methods for stable transformation of Bilharzia parasites (*Schistosoma* Spp.) and to the use of the resulting stably transformed parasites as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

Genetic engineering for producing desired gene products is presently the sole means available for producing, rather than extracting or isolating from natural sources, moderate to large size proteins in quantity. This is especially true for proteins of therapeutic importance such as, for example, hormones, enzymes, receptors, antigens and cytokines.

The quantity required as well as the required antigenic compatibility to the recipient of these products dictates the use of recombinant DNA technologies. Introducing the desired genes to host cells *in vitro* with appropriate upstream control elements has been used for the production of medically important proteins. Recombinant therapeutic proteins initially prepared in bacteria are increasingly being produced in yeasts, and even more so in mammalian cells for obtaining suitable post-translational glycosylation where it is required for a fully functional product[1,2,3].

Most of the products of recombinant DNA technology are proteins which cannot be orally administered for therapy. In general they are unstable and poorly absorbed from the gastrointestinal tract, thus they must be administered by injection, which limits their use.

A number of drug delivery systems are being developed to overcome this problem, including implantable devices that release medication over a prolonged period of time in, what is known in the art as, a slow release regime.

Gene transfer has gained a special importance in this context since genes can be inserted into the cells of animals or human patients where they could function, as therapeutic agents. This has led to experimenting

the use of several viral vectors for introducing functional genes into cells extracted from patients, and subsequent implantation of these transformed cells for *in vivo* delivery of the desired therapeutic product, in what is known in the art as *ex vivo* gene therapy [1,2,3].

The use of gene transfer, for a variety of research, medical and veterinary purposes, will be greatly facilitated if a universal transplant exhibiting long survival, and bearing no risk of malignant transformation, can be easily tailored for *in vivo* production of a desired gene product, and can be easily introduced, removed and reintroduced. Eukaryotic parasites exhibiting these features are the subject of the present invention.

Parasitism has evolved as an adaptation to life in a specialized ecological niche offered by another organism, the host, and co-evolution made parasites highly adapted to life in their host. Parasites can be found within every life form, from bacteria and plants to farm animals, laboratory animals and humans.

A spectrum of interrelations exist between parasites and hosts, ranging from a violent encounter where the parasite inflicts pathological effects and even death on its host, to a benign interaction of a non-violent mutual existence and even interdependence between host and parasite.

Such mutual existence on the genome level, as between the DNA of certain viruses and the genome of their host, has been exploited for enabling gene transfer.

For example, murine retroviral vectors, which are stably integrated into the hosts' genome, have been used in human gene therapy trials, but since they integrate with the host genome at random they can potentially induce malignancy or deleterious insertional mutations[4].

Therefore, other integrating viruses like the Adeno-associated virus vectors (AAV) are being developed for non-random integration[5]. Since integration into the host genome requires that the host cells are dividing, other systems which may be suitable for non-dividing host cell are being developed for gene transfer, adenoviruses being a notable example[6,7].

While the study of many different viruses for development of recombinant vectors for gene therapy of humans, animals and plants, is a mainstay in gene transfer/gene therapy research[1], one should consider that a long term association between host and parasite is not unique to certain viruses but also exists with much higher taxa.

Yet, the potential of unicellular or even multicellular eukaryotic parasites, for a long term production of therapeutic gene products *in vivo* has yet not been studied or exploited at all.

The potential of parasitic worms for this purpose is of particular interest because of their following advantageous features: (i) they exist in their host as separate genetic entities with independent capacity for synthesis of natural gene products, and potentially also of products of newly introduced genes; (ii) they have the qualities of universal transplants and sometime of a prolonged survival in their definitive hosts; (iii) they can be found in a variety of locations in the body where therapeutic gene products can exert their beneficial effect locally or systemically; (iv) they do not multiply as adults in their definitive host (in order to multiply their eggs have to reach the environment or intermediate hosts for further development), and therefore it is possible to control their burden in the definitive host by controlling the number of the infective stages to which it is exposed, and hence to control the amount of gene products secreted; (v) some species of parasitic worms multiply clonally as larvae in their intermediate host, which makes possible easy propagation of transgenic forms which have incorporated genes expressing desired products; (vi) elimination of the pathogenic qualities of parasitic worms is, in some cases, readily possible; (vii) elimination of the capacity of parasitic worms to continue their life cycle and cause contamination of the environment with infective forms, can be readily achieved in some cases, and in other cases can be expected, pending further studies; and (vii) removal of parasitic worms by effective drugs is possible in many cases.

All of the above features and more are shared by certain flatworms, belonging to the Genus *Schistosoma* (Bilharzia parasites), members of the Class *Trematoda*. Schistosomes are blood fukes of man and animals. Eggs of these parasites are released in the excreta and, when reaching freshwater, release the first stage larva, the miracidium, which infects certain species of freshwater snails with high host specificity. At the site of penetration the miracidium rapidly transforms to a mother sporocyst within which daughter sporocysts develop asexually. About two weeks later, multiple daughter sporocysts are released. They migrate to the snails digestive gland (hepatopancreas), and within them the cercariae, larvae infective to respective vertebrate hosts, develop asexually and are released in large numbers to the water.

An infected snail may release cercariae from about 5 weeks after infection (depending on water temperature) throughout the snail's life time (several additional weeks)[8].

As further detailed hereinunder, Schistosomes were selected as a model parasite of choice for gene transfer because of their multiple advantages. For this purpose the relevant information required for evaluating the feasibility of stable transformation and expression of foreign proteins by schistosomes and for developing, accordingly, the construct vectors and gene transfer conditions required for this purpose was integrated. The transgenic schistosomes were developed to serve as a model platform intended for the delivery and stable expression of a variety of desirable gene products in their intended host.

PCT Application No. US96/15083 by Miller, published as WO 97/11191, teaches a method of producing schistosomes as an intermediate transgene vector for secretion of desired gene products into the bloodstream of a host. The method disclosed is, however, based solely on microinjection of schistosome eggs, whereas no experimental support for the applicability of microinjection for genetically transforming schistosome eggs is provided. While reducing the present invention to practice, as is further detailed in the Examples section that follows, extensive efforts were made to genetically transform schistosome eggs by microinjection of genetic material therein, however, not even a single transformant was recoverable by this procedure. On the other hand, extensive work and calibration were required to develop transformation methods which successfully apply to schistosomes. Thus, WO 97/11191, fails to teach one of ordinary skills in the art how to genetically transform schistosomes.

SUMMARY OF THE INVENTION

The present invention provides transgenic eukaryotic parasites for use as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

According to one aspect of the present invention there is provided a eukaryotic diploid multicellular parasite transformed with a transgene.

According to another aspect of the present invention there is provided a method of providing a eukaryotic host with a protein or polypeptide, the method comprising the step of infecting the eukaryotic

host with a eukaryotic diploid parasite transformed with a polynucleotide sequence encoding the protein or polypeptide.

According to another aspect of the present invention there is provided a method of genetically modifying a eukaryotic diploid parasite, the method comprising the step of transforming the eukaryotic diploid parasite using a group transformation method.

According to still further features in the described preferred embodiments the group transformation method is selected from the group consisting of electroporation, chemical transformation, lipofection and biolistic bombardment.

According to still further features in the described preferred embodiments the polynucleotide sequence is a transgene.

According to still further features in the described preferred embodiments the protein or polypeptide is secreted from the parasite.

According to still further features in the described preferred embodiments the infection is by a plurality of individuals of the parasite, all of the individuals are of a single sex.

According to still further features in the described preferred embodiments the single sex is selected from the group consisting of male and female.

According to still further features in the described preferred embodiments the parasite is a worm.

According to still further features in the described preferred embodiments the worm is a flat worm.

According to still further features in the described preferred embodiments the flat worm is a trematode.

According to still further features in the described preferred embodiments the trematode is a schistosome.

According to still further features in the described preferred embodiments the schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis*.

According to still further features in the described preferred embodiments the host is human or animal and the parasite is infective to the human or animal.

According to still further features in the described preferred embodiments the parasite is sterile.

According to still further features in the described preferred embodiments the parasite is sensitive to a known drug, the drug is therefore effective in removing the parasite from the host.

According to still further features in the described preferred embodiments the polynucleotide sequence is integrated in the parasite's genome.

According to still further features in the described preferred embodiments the integration is by homologous recombination into a selected genomic locus.

According to still further features in the described preferred embodiments the selected genomic locus is a repetitive sequence.

According to still further features in the described preferred embodiments the selected genomic locus is a unique sequence.

According to still further features in the described preferred embodiments the parasite has distinguishable sexes, whereby a single sex of the sexes is used for the infection.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a transgenic eukaryotic parasite for use as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals. The transgenic eukaryotic parasites and their use as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals are enabled by the development of the first operable transformation procedure for multicellular eukaryotic parasites.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this

regard, no attempt is made to illustrate details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a schematic representation of the glutathion-S-transferase (GST) gene from *Schistosoma mansoni*, wherein TATA indicated the location of the promoter, ex = exon, intr = intron, PstI is a site for the *Pst*I endonuclease and GST-1 and GST-2 denote the location of these respective PCT primers;

FIG. 2 is a schematic representation of a GST-GFP fusion construct, wherein TATA indicated the location of the GST promoter, ex = exon, intr = intron, the streaked PstI is a former site for the *Pst*I endonuclease which was destroyed by blunt-end formation during the cloning procedure;

FIG 3. is a schematic representation of an GFP-Sm1-7 construct which includes the GST promoter region (TATA);

FIG. 4 is a full sequence of the GFP-GST recombinant vector including the GST-GFP fusion construct of Figure 2 (SEQ ID NO:1);

FIG. 5 is a map of the GFP-GST recombinant vector including the GST-GFP fusion construct of Figure 2;

FIG. 6 is a full sequence of the recombinant GFP-Sm1-7 vector including the GFP-Sm1-7 fusion construct of Figure 3 (SEQ ID NO:2);

FIG. 7 is a map of the GFP-Sm1-7 recombinant vector GFP-Sm1-7 fusion construct of Figure 3;

FIG. 8 shows results of PCR with cercariae, by employing GFP primers, wherein M = size markers, A = transgenic cercariae with GFP introduced inversely (control), B = transgenic cercariae with GFP introduced directly, WT = wild type cercariae and P = the GFP-GST recombinant vector of Figure 5;

FIGs. 9a-d are confocal microscopy images of cercariae with incorporated GFP-GST vector, and with control, wherein Figures 9a and 9b show GFP-positive cercariae, fluorescence (yellow-white) is seen over a red background, Figure 9c shows positive signals in transgenic cercariae fluorescence (red) in combination with Nomarski microscopy, whereas, Figure 9d shows several wild type cercariae with negative signal (only the red background is visible);

FIGs. 10a-d are confocal microscopy images of cercariae with incorporated GFP-Sm1-7 vector (Figures 10a-c) and controls (Figure 10d);

FIGs. 11a-i are confocal microscopy of transgenic adult worms and of controls, wherein Figures 11a-d show s worm from an experiment in which GST-GFP (inverse) was employed, Figure 11e shows a wild type worm, and Figures 11f-i show a worm from an experiment where the GFP was at the direct position.

FIGs. 12a-f are confocal microscopy of transgenic adult worms and of controls, wherein Figure 12a shows a strongly positive male-female pair from a GFP-GST transgenesis, Figures 12b-c show a positive male worm from an Sm1-7 transgenesis experiment, Figures 12d-e shows two negative male-female wild type worms, whereas Figure 12f shows a female wild type worm; and

FIGs. 13a-d show confocal microscopy images obtained after staining with anti-GFP fluorescent antibodies, wherein Figures 13a-b show bodies of GFP-GST transgenic cercariae, one with Cy-5 fluorescent antibody staining (red over blue, Figure 9b), and the other with both GFP (green) and Cy-5 fluorescent antibody (red) staining (Figure 9a), and Figures 13c-d show cercariae from an experiment with GFP-SM1-7 transgenesis, stained by both GFP fluorescence and CY-5 fluorescence.

FIG. 14 is a graph demonstrating shedding of cercariae from snail exposed to post electroporation of free swimming miracidia by a GST-GFP construct. Five independent experiments are shown.

FIG. 15 show PCR amplification results obtained with free swimming cercaria from various electroporation transformation experiments. Lanes: 1 - DNA extracted from wild type cercaria (negative control) ; 2 - DNA extracted from cercaria transformed with GFP is inverse position; 3 - DNA extracted from cercaria transformed with GST-GFP; 4 - DNA extracted from cercaria transformed with Sm-GST; 5 - DNA extracted from cercaria transformed with GST-GFP (GFP inverse position); 6 - DNA extracted from cercaria transformed with GST-GFP; 7 - DNA extracted from cercaria transformed with GST-GFP (GFP inverse position); 8 - DNA extracted from cercaria transformed with GST-GFP; 9 - DNA extracted from cercaria transformed with GFP-GST; 10 - GST-GFP plasmid (positive control); 11 - no DNA (negative control).

FIG. 16 show PCR amplification results obtained with DNA extracted from adult worms. Free swimming miracidia were transformed by electroporation and served to infect snails. Cercaria obtained from transfected snails were used to infect mice, from which adult worms were recovered, an analyzed. Lanes: 1 - Sm-GFP, 5 males; 2 - GST-GFP (inverse), 5 males; 3 - GST-GFP (inverse) 5 females; 4 - GST-GFP, 5 males; 5 - GST-GFP, 5 females; 6 - wild type, 5 females; 7 - wild type, five males; 8 - GST-GFP plasmid (positive control); 9 - no DNA (negative control).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of transgenic eukaryotic parasites such as parasitic worms, e.g., schistosomes, which can be used as universal grafts for *in vivo* delivery of beneficial gene products in humans and in animals.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Thus, according to one aspect of the present invention there is provided a eukaryotic diploid multicellular parasite transformed with a transgene.

As used herein in the specification and in the claims section below, the term "eukaryotic diploid" refers to organisms having a diploid set of chromosomes. It will be appreciated that, by definition, all eukaryotes are diploid in at least a part of their life cycle, typically throughout their life cycle.

As used herein in the specification and in the claims section below, the term "multicellular" refers to organisms having differentiated cell types interacting there amongst to form a functional organism.

As used herein in the specification and in the claims section below, the term "parasite" refers to an organism which, in at least a part

of its life cycle, lives on or within another species, from which it obtains nutrients and/or shelter.

As used herein in the specification and in the claims section below, the term "transformed" also means "genetically modified" and refers to the result of a process of inserting heterologous nucleic acids into the genome of a species. Transformation may be effected according to the present invention by group transformation methods, such as electroporation, chemical transformation, lipofection or biolistically via particle bombardment. However, as is further exemplified in the Examples section that follows, transformation cannot be effected according to the present invention by microinjection, which is an individual transformation method. As is further exemplified hereiunder electroporation, which is a group transformation method, was successfully employed to stably transform miracidia, the first schistosome larval stage, whether still in ova or after hatching.

The above listed group transformation methods are believed to be useful, because all these methods impose fewer damage to the organism or developmental stage thereof undergoing transformation, as is compared to microinjection. Furthermore, the general practice of the above listed group transformation methods is well known in the art, whereas calibrating a method for maximal efficiency is in the ability of an ordinary artisan. Such group transformation methods are generally described in, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York 1989, 1992; in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Maryland 1989; Chang *et al.*, Somatic Gene Therapy, CRC Press, Ann Arbor, MI 1995; Vega *et al.*, Gene Targeting, CRC Press, Ann Arbor MI (995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston MA, 1988; and Gilboa *et al.*, Biotechniques 4 (6): 504-512, 1986. It is believed that one of ordinary skills in the art would be able to adapt a most suitable transformation procedure to an organism of choice.

As used herein in the specification and in the claims section below, the term "transgene" refers to any polynucleotide sequence which is used to stably transform an organism or cells thereof. A transgene typically forms a part of an expression cassette which includes an expressible polynucleotide sequence which typically encodes a polypeptide or protein, i.e., the transgene. In addition to the nucleic acid

encoding the protein or polypeptide of choice, the cassette of the present invention may include one or more of the following genetic elements: a selectable marker, an origin of replication, cis acting control elements such as a transcriptional promoter and an enhancer, a translation start site, a polyadenylation site, a signal sequence for secretion of the protein product and the like. The appropriate assembly of these elements into an operative expression cassette is within the skills of an ordinary artisan. When a transgene is integrated into the genome of a parasite using homologous recombination close to control elements at the site of integration, these control elements may direct the expression of the coding region of the transgene.

According to another aspect the present invention provides a method of providing a eukaryotic host with a protein or polypeptide. The method according to this aspect of the invention is effected by infecting the eukaryotic host with a eukaryotic diploid parasite transformed with a polynucleotide sequence encoding the protein or polypeptide. Since many parasites are known to have restrictive species specificity, the eukaryotic diploid parasite and the eukaryotic host are selected compatible, in other words, the parasite is selected infectious to the host.

As used herein in the specification and in the claims section below, the term "eukaryotic host" refers to a eukaryotic organism from which a parasite obtains nutrition and/or shelter.

According to a preferred embodiment of the present invention the protein or polypeptide is secreted from the parasite. To this end, the polynucleotide sequence preferably includes signal peptides for secretion. However, non-secreted proteins are also within the scope of the present invention since parasite death can cause release of the protein to the host's circulation.

The protein may be any desired protein for which a gene has been isolated. Typically the protein of choice is missing, dysfunctional or ineffective in the host, whereas the parasite serves to replenish the missing, dysfunctional or ineffective protein. The protein may be a hormone, a growth factor, an enzyme, a clotting factor, a cytokine, an antigen, a receptor, a proteinaceous anti-microbial molecule, a proteinaceous neuro-transmitter, etc.

For example, the protein may be insulin for treatment of insulin dependent diabetes. The protein may be a growth hormone or sex hormone for treatment of conditions where any of these hormones is

absent, dysfunctional or ineffective, or any other hormone of impaired expression in the host.

In order to control parasite burden within the host and to minimize the risk of environmental parasite pollution it is advantageous in some embodiments to infect the host with a known number of individual parasites of a single sex, either male or female, so as to avoid reproduction.

Alternatively, the parasite burden is maintained un-increased by infection with sterile (non-reproducing) parasite individuals. Sterilization may be accomplished genetically, for example by crossing heterozygotes for a recessive sterility gene, physically by removal of sex and/or reproduction organs or by irradiation or chemical treatment known to damage these organs.

Burden selection depends on various factors, including, but not limited to, the amount of gene product secreted by the parasite and the amount of gene product required by the host for reversing symptoms or treating a condition. One ordinarily skilled in the art would know how to adjust the parasite burden by monitoring the host's symptoms.

Many parasitic worms are known. These includes species belonging to certain classes of the phyla *Platyhelminthes*, *Acanthocephala* and *Nematoda*.

According to a preferred embodiment of the present invention the transgenic parasite is a worm, preferably a flat worm, preferably a trematode, most preferably a schistosome, including the species *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis*.

For treatment of animals, such as house hold and farm animals and animals kept in captivity a parasite compatible with that animal is selected, whereas for treatment of man, a parasite compatible with man is selected, all according to the species specificity of the parasite. One ordinarily skilled in the art would know how to select a parasite species which is compatible to treat a given host.

According to a preferred embodiment of the present invention the parasite is sensitive to a known drug, the drug is therefore effective in

removing the parasite from the host when so desired. For example, schistosomes are sensitive to a variety of schistosomicidial drugs, including, but not limited to, praziquantel, oxammquin, metrifonate, hycanthone, nicrosamide and other. However, drugs for other parasites are well known in the art.

According to a preferred embodiment of the present invention the polynucleotide sequence (transgene) is integrated in the parasite's genome. The integration may by effected by homologous recombination into a selected genomic locus, such as a repetitive sequence or a unique sequence. Integration by homologous recombination is presently preferred since the chances of hampering genes which are important or crucial for the functionality of the parasite in general and within the host in particular are reduced. Integration into a repetitive sequence is advantageous since multiple harmless integration sites are available, increasing both the chances of integration and the number of integration events, however, these sites are disadvantageous in that in some cases they are located in non-transcribed regions of the genome. Integration by homologous recombination into unique sequences is also within the scope of the present invention. These sequences are selected according to their functionality. In order to obtain high level of expression of the transgene, the integration site is preferably selected close to and down stream of strong and effective expression control sequences. In any case, the scope of the present invention is not limited to integration by homologous recombination, in other words non-specific or non-targeted integration is also within the scope of the present invention, such that even parasites for which no sequence information is available can be transformed. Furthermore, non integrated or extrachromosomal (e.g., episomal) transgenes are also within the broad scope of the present invention, although at present less favorable due to the possibility of diluting or loosing the transgene during cell divisions.

While reducing the present invention to practice, schistosomes served as a choice model system demonstrating the potential use of eukaryotic parasites in general for *in vivo* delivery of desired gene products in their host. The use of parasitic worms is presently of choice. This may include tapeworms such as *Hymenolepis diminuta* for introducing gene products into the intestines, and the filaria *Brugia malayi* for introducing gene products into the lymphatics, and into the circulation. Conditions for abrogating pathogenicity and transmission

may influence parasite selection. According to the present invention transgenic schistosomes are employed where unisexual infections provide these requirements.

When foreign genes were introduced by electroporation into miracidia they were incorporated and passaged to schistosome larvae developing in the snail, and subsequently into adult worms developing in the vertebrate host. Stable transformation and development of transgenic worms expressing desired transgene product (GFP in the present work which served as an example of a foreign gene) in the target recipient of this delivery system has been achieved.

The results achieved while reducing the present invention to practice demonstrate that introduction of foreign genes into schistosome miracidia within ova or free swimming after hatching from the ova is applicable, and that a high proportion of transgenic cercariae was subsequently obtained after clonal multiplication in the snail host as expected.

The expected advantage of introducing the foreign DNA into miracidia by group transformation methods, e.g., by electroporation, was confirmed. Introducing the foreign genes both into the GST gene and preferably into the SM1-7 repeated sequence of the schistosome's genome were successful although the second location seems more suitable for obtaining better cercarial and adult worm yields.

The present invention is designed to provide the conditions for stable transformation of schistosomes and a modular system into which desired genes and control elements can be introduced. In the examples provided herein, the foreign genes were reporter genes introduced into the schistosomal genome preferably by homologous recombination. It is demonstrated that control elements of a schistosomal gene such as GST can be employed for expressing the foreign genes, and that the foreign genes can be introduced into an established (unique) schistosomal gene or into highly repeated DNA sequences of satellite DNA. The use of alternative strong promoters which have been previously demonstrated to be active in transgenic eukaryotes, such as the SV40 promoter[19], but not excluding other promoters, can also be considered for the same purpose. Targeting of the foreign genes into genomic locations other than the GST gene and the Sm1-7 repeated sequence can also be considered. One takes into consideration that in some cases the transfected gene may be toxic in higher concentrations, or that its expression will require external

regulation. In these cases constructs with inducible promoters can be prepared. The most effective inducible promoters that are in use for transformations of eukaryotic cells are promoters with tetracycline regulatory systems[68]. Addition or depletion of tetracycline causes these promoters to start or stop transcription of the corresponding downstream gene coding regions. Other triggers of inducible promoters can be incorporated as they become available. The introduction of the construct containing the foreign gene into a satellite DNA region offers a possibility to avoid putative damage that may arise from introduction of the foreign gene within a single copy gene whose function is essential for the survival of the parasite. The stable introduction of foreign DNA into the schistosomal genome enables stable expression throughout its life cycle, depending on the control elements of choice. This, however, does not exclude introduction of the desired genes as episomes including the desired gene in a recombinant vector. It also does not exclude introduction of the desired foreign genes into other locations such as mitochondria[69], intra-cytoplasmic locations, and artificial chromosomes[70-71], as well as into other possible locations not mentioned here. The high efficiency of introducing foreign genes into the miracidium while in the ovum or when free swimming after hatching is demonstrated herein below. Electroporation proved suitable for introducing foreign genes into schistosomes, but biolistics, should be suitable for this purposes too as can be expected from previous work with eukaryotic cells[15]. Other methods such as lipofection and chemical transformation , but not excluding additional methods, are also possible now that the principles and feasibility of producing transgenic schistosomes have been demonstrated. Introduction of the foreign DNA into the miracidium while it is enclosed within the ovum or free swimming after hatching was selected for this work but this does not exclude introducing the DNA at other developmental stages. The non-motile primary sporocyst, which can be obtained *in vitro* directly by transformation from miracidia[72] is a very likely stage into which foreign genes can be introduced and which can then be transplanted directly into the snail host as is done with daughter sporocysts taken from infected snails[29]. Since schistosomes multiply clonally within their intermediate host (the snail), introduction into miracidia or sporocysts and then infection of the snail (naturally for miracidium or artificially for the sporocyst) should enable expansion of transgenic clones. Infection of

the snail by a single miracidium or sporocyst will ensure a single-sex progeny of cercariae for a long period of shedding (weeks). Once the transgenic nature of the cercariae as well as their sex[59] are determined by PCR with appropriate sex chromosome specific primers, and gene products identified by, for example, an adjoining reporter gene, and or by specific detection of transgene products, the clone can be expanded to any desired size by transplantation of the transformed sporocysts into naive snails[29]. In this way "farming" of desired transgenic clones can be accomplished and cercariae thereof can be taken "from the shelf" at any time for introduction into a few or into many target animals or humans at the same time.

Abrogating the parasites' pathological responses is a crucial requirement for employing parasites for delivery of desired gene products. It is therefore important to analyze carefully the dynamics of schistosome infection and disease. The best known model for this purpose is *S. mansoni* infection in laboratory rodents and the following summary is a generally accepted overview[73]. *S. mansoni* worms develop to maturity (and egg laying capacity) within five weeks. Eggs in the tissues require one week to mature and then survive for three weeks. During the first five weeks of infection Th1 cells are dominant in the murine immune response with INF-γ and IL-2 as dominant lymphokines. After egg laying begins, Th2-type responses become dominant with IL-4, IL-5 and IL-10 as dominant lymphokines. IL-4 secretion is associated with IgE response and IL-5 with eosinophilia. IL-10 down regulates the Th1 responses. At about 12 weeks of infection immunological down-regulation occurs, accompanied by down regulation of the granulomatous response around *S. mansoni* eggs and by a reduced collagen synthesis.

At the early stages of infection[8], acute symptoms of fever, diarrhea, abdominal pain, myalgia, arthalgia, emaciation, cough, hepatomegaly and urticaria were the most frequent symptoms reported[74,75]. Immune complexes appear to have an important role in the acute disease, and its resemblance to serum sickness (resulting from the well established therapeutic use of antitoxines) has been noted[76,77].

In the baboon, which has been proposed as a model for acute schistosomiasis mansoni, the onset of symptoms coincides with the onset of egg laying[47]. In the mouse too, early lesions induced by mature eggs appears as the major factors in the pathogenesis of acute schistosomiasis mansoni[46].

In humans, on the other hand, in a few cases of those reported with a known time of onset of infection, symptoms of acute disease were said to have started even before egg laying[74,75]. These findings, although limited, raise the question of whether transgenic unisexual schistosomes may in some cases cause acute disease symptoms in humans (as opposed to animals).

Consideration of this possibility is therefore required when initial trials of the new technology in humans are carried out. The limited information so far available[74,75] is not clear-cut about the exact time of exposure to infection in humans, because the time of exposure was determined based on questionnaires and epidemiological information. Also, the possibility that the symptoms were caused by other, coinciding, causative agents was not excluded, and finally, even if pre-oviposition symptoms of acute schistosomiasis did occur in humans, it is not possible under natural conditions to separately characterize them (in terms of nature, severity and duration) from post-oviposition symptoms. Only actual infections of humans selected for treatment by unisexual transgenic parasites will make it possible to clarify the net pathogenicity of worms. It is also important to mention in this context that egg antigens and antibodies to egg antigens passed from mothers to fetuses were suggested to protect against acute disease[78]. This finding reinforces the presumed role of the eggs in the pathogenesis of acute disease in humans too, and it also suggests that a suitable immune manipulation may abrogate acute disease (if eventually found in controlled unisexual human infections).

The present invention is suitable for *in vivo* release by the parasites of a variety of desired products of transgenes. It will enable preparation of parasites carrying more than one transgene, or introduction of more than one population of transgenic schistosomes into target hosts for multiple effects, when so necessary.

It will primarily enable introduction of transgenic larvae (cercariae or schistosomula) which will complete development in the recipient within several weeks. But transplantation of fully developed worms grown in animals for exerting an immediate effect will also be possible, but will be logistically more difficult. Among the gene products that can be delivered by the present invention are the following, not excluding others not mentioned, hormones and growth factors, enzymes, clotting factors, cytokines, antigens, receptors, anti-microbial

molecules, neuro-transmitters, etc. These can be used in three main biomedical/animal sciences domains as follows.

For preparing laboratory animals presenting features for basic scientific research purposes: Over- expression of desired gene products; expression of inhibitors of natural gene products; correction (total or graded) of the phenotypic outcomes of knocking out genes through breeding or genetic engineering; altered susceptibility to disease conditions that may be caused by agents such as, but not limited to, pathogens, chemicals, pharmaceuticals, natural products, environmental factors, etc.; altered fecundity and embryonic development; altered behavior; etc. Since *S. mansoni* which was used in this work readily infects mice, this embodiment of the present invention is suitable for immediate application.

For veterinary purposes: Over-expression of desired gene products; expression of inhibitors of natural gene products; correction (total or graded) of genetic or acquired deficiencies; altering the rate of development and of gaining body mass; altering fecundity including sperm count and oestral cycle; altering efficiency of milk production; altering the rate of production of transgene products in transgenic farm animals (e.g., in milk) and in clones thereof; altering susceptibility to disease agents such as microbes, but not excluding other agents; etc.

For medical purposes: Restoration of deficiencies whether genetic[1] or acquired, such as, but not limited to, hormonal deficiencies (for example, deficiencies in growth hormone and insulin), metabolic deficiencies (for example, deficiency in metabolic enzymes), hematological deficiencies (for example, deficiencies in clotting factors), immunological deficiencies (for example, adenosine deaminase (ADA) deficiency), etc.; immunotherapy (including vaccination requiring long term antigenic stimulation, and over production of cytokines); anti-microbial therapy (including the anti-viral action of interferon, and microbe-binding soluble receptors for eliminating disease agents such as HIV); anti-cancer therapy; treatment of drug addiction; treatment of a variety of poisoning conditions; amelioration of geriatric conditions; etc. Since *S. mansoni* is a parasite of man this embodiment of the present invention is suitable for immediate clinical practice.

The procedure proposed in accordance with the teachings of the present invention for prospective uses in animal sciences, should enable obtaining phenotypic features of the target animals, which otherwise

could be achieved only by the complex, time consuming and much more expensive breeding or genetic engineering/transgenesis for each single property in each host strain.

It should make treatment of humans with *in vivo* transgenes, universally compatible and therefore much more readily available and inexpensive.

In addition, the procedure disclosed avoids genotypic alteration of patients, thus avoiding the accompanying risks of mutagenesis and malignant transformation.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

EXAMPLE 1

Theoretical and Experimental Considerations

This preliminary part of the work involved assembly of data into an integrated theoretical concept for preparing transgenic schistosomes intended for *in vivo* delivery of desired gene products in their host's circulation. This part of the work led to the formulation of relevant working hypotheses, and to relevant experimental designs. In the core of this integrated concept are the putative advantages of schistosomes for the proposed purposes, as follows:

Introduction of foreign genes into schistosomes should be very easy, and much safer than direct gene transfer to humans: Schistosomes are separate genetic entities within their host. Thus making them transgenic for expressing desired genes within the host, should be much safer than introducing foreign genes directly into the host's genome, a manipulation which may result in malignant transformation or insertional mutations harmful to the host[1]. Gene transfer into worms has

so far been successful only in the free living non-parasitic nematode *Caenorhabditis elegans*[9]. The success in this case is due to the simple and rapid (a few days) life cycle of this organism. Thus, injection of recombinant expression vectors into the oocytes within the ovarium resulted in a proportion of transgenic larvae which developed into fast-multiplying transgenic adults. This approach is less likely to succeed in schistosomes because the *in-vitro* culture of adults with subsequent egg laying and maturation of an infective miracidium of this obligate endo-parasite is complex[10], and the life cycle of schistosomes is long (requiring about 3 months to be completed in the snail and vertebrate hosts). Since vigorous asexual (clonal) multiplication of schistosome larvae occurs within the snail[8], it would be best to achieve a high proportion of transgenesis of parasites just before they start to multiply within the snail so that transgenic organisms will be amplified during intramolluscan multiplication (see below). Miracidia, schistosome larvae which infect snails by active penetration, can therefore be considered the prime target for transgenesis. The miracidium is a ciliated multicellular organism with 4 epidermal plates arranged in 4 tiers and covered with cilia and apical musculature and glandular structure suitable for penetrating the snail host. Most of the posterior 1/3 part of the organism is filled by a cluster of germinal cells which are interconnected and are also connected to the surface of the organism[11]. Germinal cells undergo multiplication shortly after the miracidium penetrates into the snail and transforms into a mother sporocyst[8,11], which is the start of asexual multiplication to form daughter sporocysts and subsequently cercariae[8,11]. In addition, miracidia are highly acidiophilic[11] and are located in highly perforated ova[12] capable of allowing passage of macromolecules[13]. These features combined (in particular the connection between germ cells among themselves and with the surface of the organism, as well as their acidophilicity[11]), seem theoretically favorable for introducing DNA into miracidia. Hitting miracidia with a wide scatter of DNA is a likely approach for successful introduction of DNA into their germ cells. Targeting miracidia while they are within eggs seems a more reasonable approach since the highly motile free miracidia in water may escape an effective hit or may be relatively more vulnerable. However this limitation does not apply when the method of transformation involves maintaining a high concentration of DNA intended for introduction around the miracidia. Introduction of the

foreign DNA may be achieved by electroporation[14], or by biolystics[15]. Chemical transformation[16] by $(Ca)_2(PO_4)_3$, $CaCl_2$ or the like can also be considered if miracidia will not be adversely affected by these chemicals, and lipofection[17] (by using micelles or liposomes containing DNA) may also be suitable. As is further detailed below, it was chosen to transform schistosome miracidia within their eggs or after release from the eggs (free swimming) by electroporation, and to use the putative transgenic miracidia for infecting snails, and then to establish that transgenic cercariae and subsequently transgenic adult worms expressing products of the transgene, can be recovered.

Schistosome developmental stage suitable for transformation: The maintenance of schistosome cultures in the laboratory is a common practice (see experimental section below) and a variety of life cycle stages can be harvested. Foreign genes are introduced into the schistosome germ cells, which are responsible for larval development and multiplication. Germ cells are present in miracidia, sporocyst, cercariae, and young worm (schistosomula) and in sexual organs of adult worms. When transformation into germ cells takes place before clonal multiplication within the snail host, any one transfected larva serves as a source for numerous transgenic cercariae, each of which can develop to a transgenic adult worm. Miracidia, schistosome larvae, which are released from fully developed eggs and infect snails by active penetration, are the main proposed target for transgenesis. The reason for this choice is explained below. Miracidia are taken for transformation when still within eggs or, alternatively, after they are released from the eggs by hatching under hypotonic conditions. The selection of miracidia for transformation is based both on their position in the life cycle prior to clonal multiplication, as well as on their anatomy which favors introduction of foreign genes into germ cell as explained below. Large numbers of miracidia can transform *in vitro* to large number of primary sporocysts which are also candidates for transformation as is further detailed below.

The miracidium is a ciliated multicellular organism with four epidermal plates arranged in four tiers and covered with cilia and apical musculature and glandular structure suitable for penetrating the snail host. Most of the posterior third portion of the organism is filled with a cluster of germinal cells, which are interconnected and are also connected to the surface of the organism[11]. Germ cells undergo multiplication

shortly after the miracidium penetrates into the snail and transforms into a primary (mother) sporocyst (Pan CT. Studies on host-parasite relationship between *Schistosoma mansoni* and the snail *Australorbis glabratus*. Am. J. Trop. Med. Hyg. 14:931-976, 1965), which is the starter of asexual multiplication to form daughter sporocysts and subsequently cercariae which infect the vertebrate host and develop into adult worms. In addition, miracidia are highly acidophilic (Pan CT. Studies on host-parasite relationship between *Schistosoma mansoni* and the snail *Australorbis glabratus*. Am. J. Trop. Med. Hyg. 14:931-976, 1965) and are located in highly perforated ova (Stenger RJ, Warren KS, & Johnson EA. An ultra structural study of hepatic granulomas and schistosome eggshells in murine hepatosplenic schistosomiasis mansoni. Exp. Mol. Pathol. 7: 116-132, 1967) capable of allowing passage of macromolecules (Hangh LM et al. The *Schistosoma mansoni* egg granuloma: Antigenic secretions and the etiology of egg granulomas in mice. Exp. Parasitol. 35: 288-298, 1974). These features combined, and in particular the connection between germ cells among themselves and with the surface of the organism, as well as their acidophilicity, are favorable for introducing nucleic acids into miracidia. Mature eggs containing fully developed miracidia are suitable for transformation because of the anatomy of the fully developed miracidium within them as described above. Schistosome lay immature eggs, which undergo development to fully embrionated eggs within a few days after they have been laid (Pellegrino J & Faria J. The organ method for the screening of drugs in schistosomiasis mansoni. Am J Trop Med Hyg 14:363-9, 1965). Therefore, collection of mature schistosome ova for transformation needs to be done from tissues of an infected animal where eggs become trapped, such as the intestines and livers of infected mice. A large proportion of such eggs is fully embrionated (Hamburger *et al.* *Schistosoma mansoni* soluble egg antigens: determination of the stage and species specificity of their serologic reactivity by radioimmunoassay. J Immunol 117:1561-6, 1976). Collecting large amounts of eggs is essential for group transformation. Naked miracidia with their cytoplasmatic cover and cluster of germinal cells which are interconnected and are also connected to the surface of the organism[11] are also suitable targets for group transformation, because of the accessibility of foreign DNA to the germ cells. They can be obtained in large numbers by hatching of eggs collected from tissues of infected

animals. A successful transformation of the eggs or of miracidia depends on application of a suitable transformation method. Primary sporocysts are also proposed for group transformation. They can be obtained in large number for this purpose by *in vitro* transformation of miracidia (Lodes MJ & Yoshino T. Characterization of excretory-secretory proteins synthesized in vitro by Schistosoma mansoni primary sporocysts. J Parasitol 75:853-62, 1989). Transformation of sporocysts is proposed by methods different from those proposed for miracidia (see below).

Conditions for effective transformation of schistosomes: Hitting miracidia mature eggs and miracidia, and also primary sporocysts, in group with a wide scatter of DNA is shown herein to be the sole approach for successful introduction of foreign DNA into their germ cells. A confluent distribution of DNA molecules is proposed as a preferred choice in order to ensure entrance of the DNA through the micropores of embrionated eggs, and into the extensions of germ cells which reach the surface of the miracidium (see above). This approach enables the simultaneous production of numerous transformed miracidia at once. Each of these is a source of production of a large number of larvae by asexual multiplication within the snail, and each of the transgenic progeny of cercariae has the capacity of developing into an adult worm within the definitive host (humans or animals). These quantitative aspects are essential for preparing large numbers of transgenic cercariae for the practical purposes described. In the case of primary sporocysts transgenesis can be effected, again by group transformation, using particle bombardment as described below. The transformation approach presented herein increases the chances of a successful transgenesis of schistosomes, and therefore a higher chance that sufficient numbers will withstand the damages caused by the transformation procedure and subsequent developmental restrictions.

Microinjection for introducing foreign DNA into schistosome eggs is not only not attractive, it also fails to yield transformants. The *in-vitro* culture of adults with subsequent *in vitro* egg laying and maturation of an infective miracidium of this obligate endo-parasite is complex (El Ridi, R. et al., *Schistosoma mansoni* oviposition *in vitro* reflects worm fecundity *in vivo*: Individual parasite age- and host-dependent variations. Int. J. Parasitol. 27:381-387, 1997). Second, injection of individual eggs with foreign DNA is quantitatively inefficient. Thus, high numbers transformants is required considering the long and multistage life cycle of

schistosomes, which stands ample possibilities of losing foreign genetic material at any one stage. Third, injection of mature eggs is likely to damage the miracidium because it is short lived anyway and is dependent for its viability on preservation of motility and active penetration capacity into its snail host. In any case, attempts to prepare transgenic schistosomes by injection of genetic material to schistosome eggs have so far failed.

The electroporation technique (Wong TK & Neuman E. Electric field mediated gene transfer. Biochem. Biophys. Res. Comm. 107: 584-587, 1982) specifically adapted for the purposes of the present invention is described herein as an example demonstrating the feasibility of group transformation. Any other procedure which involves immersion of schistosome eggs or miracidia in a medium containing DNA of a suitable construct for group transformation can be applied. In particular, chemical transformation (Cheng C, & Okayama H. 1987. High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7:2745-2752, 1987) by $(Ca)_2(PO_4)_3$, or by $CaCl_2$ as well as lipofection (Felgner PL *et al.* Lipofection: A highly efficient, lipid-mediated DNA/transfection procedure. Proc. Natl. Acad. Sci. U.S.A. 84:7413-7417, 1987). The electroporation method for transforming schistosome miracidia (within their eggs or after hatching) is considered the simplest and presently most available for practicing the present invention, and it therefore served experimentally to reduce the present invention to practice. These group transformation methods are used for introducing foreign DNA into cells and are less likely to operate as effectively while attempting at introducing the DNA into deeper tissues. It should therefore be emphasized that the connection of germ cells with the surface of miracidia, as well as the presence of micropores in the schistosome egg make the use of these group transformation methods highly suitable in the case of schistosome miracidia. Thus, while reducing the present invention to practice, as is further exemplified hereinunder, electroporation was used for stable transformation of schistosomes.

Particle bombardment of eggs and miracidia is considered possible but less efficient as is compared to electroporation, chemical transfection and lipofection because the latter three methods, and in particular, electroporation, involve milder driving forces and confluence of high concentrations of DNA around the target. Particle bombardment,

on the other hand, delivers DNA attached to relatively large particles. Thus, for example 1.6 mm gold micro-carriers were recently used for delivering nucleic acids into adult schistosomes for transient expression thereof (Davis RE *et al.* Transient expression of DNA and RNA in parasitic helminths by using particle bombardment. Proc Natl Acad Sci, USA 96:8687-92, 1999). Particles of this size cannot pass through the micro-pores in the schistosome eggshell and are therefore likely to require more drastic bombardment energies in order to penetrate the hard shell and enter the miracidium within the egg. A multitude of particle passing the miracidial ciliated epithelium are likely to reduce the mobility of the miracidium and therefore its ability to penetrate the snail for survival. Also, such particles covered with the DNA do not offer a confluent distribution of DNA molecules in order to enable efficient targeting to germ cells. It is therefore propose herein to employ particle bombardment primarily for transfection of primary sporocysts with the added possibility of transforming miracidia (free or within eggs). Primary sporocysts can be obtained in large numbers from miracidia and maintained *in vitro* by available methods (Lodes MJ & Yoshino T. Characterization of excretory-secretory proteins synthesized in vitro by Schistosoma mansoni primary sporocysts. J Parasitol 75:853-62, 1989). They are devoid of vigorous movement and of the capacity to penetrate into snails. Therefore, following particle bombardment, sporocysts can be implanted into snails (Jourdane J & Therone A. Schistosoma mansoni: Cloning by microsurgical transplantation of sporocysts. Exp Parasitol 50:349-57, 1980). The use of electroporation, chemical transfection or lipofection is considered less suitable for transforming sporocysts because their body cover (tegument) changes during transformation from the miracidium stage (Hockley DJ. Ultrastructure of the tegument of schistosoma. Adv Parasitol 11:233-305, 1973) and presence of external connections to the internally located germ cells have not been demonstrated.

There is a choice of genomic locations suitable for introducing foreign genes into schistosomes: Schistosomes have a diploid genome with 16 chromosomes (2n=16)[18]. The control of gene expression in schistosomes has not been sufficiently defined and a system for introducing foreign DNA into the parasite has not yet been developed. For expression and secretion of desired gene products in schistosomes it is important that control elements responsible for high degree of

expression and secretion will operate in the environment of the schistosomal cell. Many of the control elements generally employed are species specific for the target organism of transformation, so that elements working properly in a mammalian system may not be suitable for lower organisms like schistosomes. Many expression vectors utilize promoter and other control elements derived from viral genomes. For instance, LTRs from the promoter of retroviruses or SV40 can, when active, enable high levels of gene expression[19]. These may indeed be suitable for gene transfer in schistosomes too. A more specific approach is to combine the foreign gene with control elements of the target organism (schistosomes in our case) and introduce the construct into the selected location in the target genome by homologous recombination[20,21]. Thus, the expression of the foreign gene which was integrated into the target genome will be under the control of the target gene into which it was integrated. Expression will therefore be stable and the newly introduced gene will be replicated stably with the recipient genome. Several schistosomal genes have been cloned and characterized, and all the genes which express a protein which is secreted, such as Glutathion-S-transferase[22,23] Sm 31/32[24] and others[25,26, 27], seem suitable for the purpose of the present invention. With more information accumulating on schistosomal genes, more candidates for integrating foreign genes into the schistosomal genome are likely to emerge. An alternative site for introducing foreign genes into schistosomes is an abundant repeated sequence of 121 bp units tandemly arranged which was found in the genome of *S. mansoni*[28]. When introducing foreign genes into schistosomes the selection of transgenic organisms by selective markers seems less suitable than by reporter genes because selection may be hampered by the slow life cycle and the multicellular nature of the organism. Suitable reporter genes may actually enable identification of transgenic sporocysts in a dissected snail for transfer into naive snail for propagation and maintenance[29] (see below). Various reporter genes like the genes for chloramphenicol acetyl transferase (*CAT*)[30], β galactosidase (*β-Gal*)[31], luciferase (*luc*)[32], or green fluorescent protein of jellyfish (GFP)[33,34]. The latter, although less sensitive than *luc* by 3 orders of magnitude, has recently been widely used in transgenesis because its expression in a transgenic animal indicates that expression is efficient far beyond the detection limit of the

expression products. GFP was hence selected as a reporter gene in this work.

Sex selection: Schistosome males are preferably selected as carriers of transgenes for *in vivo* delivery of desired gene products. Males are larger than females, they have a larger tegumental area and their tegument is directly exposed to the exterior environment for release of tegumental sloughing or secretion products containing transgene products. The females on the other hand are slender, they have a smaller tegumental area and they are encircled in permanent copulation within a canal (gynecophoric canal) within the males. Furthermore, the female of schistosomes requires continuous contact within the gynecophoric canal of the male in order to complete its growth and maintain sexual maturity (Clough ER. Morphology and reproductive organs and oogenesis in bisexual and unisexual transplants of mature Schistosoma mansoni females. J Parasitol 67:535-9, 1981). The contact with the male involves passage of nutrients and developmental stimuli from male to female (see, e.g., Atkinson KH & Atkinson BG. Biochemical basis for the continuous copulation of female *Schistosoma mansoni*. Nature 283:478-479, 1980). While female schistosomes naturally produce and secrete gene products associated with egg production (such as Mehlis Glands and vitelline glands) these cannot be utilized because a sexually functional female is required for producing these gene products (Johnson KS et al., Possible eggshell protein gene from Schistosoma mansoni. Mol Biochem Parasitol 22:89-100). Also, artificial abrogation of egg laying in a bisexual infection involving transgenic females is likely to simultaneously interfere with the expression of transgenes associated with female reproductive gene products, such as the egg shell protein genes. The control of female specific gene expression is not sufficiently understood at this stage to enable capitalizing on these systems for expressing transgenes.

Schistosomes multiply clonally in their intermediate host, and this is expected to greatly facilitate expansion and "farming" of desired transgenic clones: Schistosomes multiply clonally in freshwater snails in a way that presents an important advantage for propagation of transgenic larvae. As already mentioned the miracidium transforms at the penetration site into a mother sporocyte within which germ balls are formed which develop into daughter sporocytes. Daughter sporocysts then emerge and migrate to the snails digestive gland, the

hepatopancreas, and germ-balls within them continuously develop into thousands of cercariae (the stages infective to man or other hosts) which continue to be shed from the snail throughout his life, generally for many weeks[8,11]. Since all subsequent larvae developing from a single miracidium are a clone, starting snail infection by exposing it to a single miracidium will result in a unisexual infection. As will be subsequently explained (see below), a unisexual infection is required to prevent the major, egg related pathology. Snails shedding cercariae of the desired sex can be selected by examining cercariae by the polymerase chain reaction (PCR) with sex specific primers[35]. Furthermore, long term maintenance of desired clones can be done by serial transfer of sporocysts (within each sporocyst a clone of cercariae is continuously produced) from snail to snail by implantation[29], thus potentially enabling maintenance of "a farm" of transgenic clones. Each clone in such a "farm" will release transgenic cercariae carrying a certain transgene, and each can be expanded as desired by implantation into additional naive snails.

The anatomical location of schistosomes, their size, and excretion of products by them are expected to be of advantage for employing them as a transgenic platform for expressing and delivering desired genes in vivo: Schistosomes reside in the mesenteric-portal veins or in the vesical plexus (depending on the species). They are capable of secreting macromolecules[36] which should be accessible into the blood, with subsequent systemic distribution. With regard to their residence in veins they are unique among parasitic worms, although access of materials secreted by parasitic worms to the hosts' vasculature also occurs among nematodes causing lymphatic filariasis which reside in the lymphatics[37]. Adult schistosomes may occasionally be found in ectopic locations[38], and may be theoretically transplanted in blood vessels of additional organs where the substances they secrete may be required for therapy. In adult schistosomes ongoing turnover of the outer membrane regularly releases membrane constituents into the blood stream[39]. Other constituents are secreted as has been described above[22-27]. These findings which demonstrate that schistosomes can release gene products into the circulation are strengthened by actual demonstration of circulating schistosomal constituents in the circulation of the host[24]. Schistosomes are about 1 cm long, but the biomass of the male is several times larger than that of the slender female, and its maturation is

independent of the presence of the female. By contrast, the slender female requires continuous contact with the male in order to complete its growth and maintain sexual maturity. The female is permanently carried by the male in a special "gynecophoric" canal and studies have shown the passage of nutrients and developmental stimuli from male to female[40,41,42]. These differential features make the schistosome male a preferred candidate for *in vivo* production of desired gene products.

The pathogenicity of schistosomes can be ready abrogated as required for their use as universal transplants: In vertebrates schistosome pathogenicity depends mainly on their capacity to lay eggs, a proportion of which do not succeed to be passed out with excreta, but become trapped in the host's tissues (of the intestines and liver or of the bladder-depending on the schistosome species). Antigens secreted from the trapped eggs induce a vigorous immune granulomatous response and subsequent fibrosis[43]. These T-dependent immune responses[44] lead to the structural and hemodynamic changes typical of the disease (schistosomiasis or Bilharzia)[8]. At conditions where eggs are not produced, like in the case of unisexual infection, even large number of worms do not cause the typical granulomatous disease, as was found in mice, the common model of egg-induced pathology in experimental schistosomiasis mansoni[45,46]. In schistosomes, where eggs cause most of the pathology, unisexual, "eggless", infection can be achieved very easily since all the cercariae shed by a snail which was infected by a single miracidium are unisexual[45] and sterilization for preventing egg laying is unnecessary. The early stage of infection by *S. mansoni* in humans has been known to cause an acute disease[8]. Whether or not eggs are an important factors in causing acute disease in humans, as is the case in murine schistosomiasis mansoni[46] and in *S. mansoni*-infected baboons[47], will be discussed. Eggs deposited in the intestines and the liver of animals and humans, infected with *S. mansoni* induce pathology in the intestines and in the liver. In chronic hepatosplenic schistosomiasis mansoni porto-systemic anastomoses may develop due to portal hypertension which develops as a result of the cumulative egg-related hepatic pathology. In such cases eggs and occasionally worms can be found in the lungs and to a lesser extent a variety of ectopic anatomic locations. The pathology in most such cases is caused by the eggs[8] but even ectopic adult schistosomes were not surrounded by inflammatory cells[38], except perhaps rarely when they die in the

lungs[48]. The pathologic effects of dying worm are probably minimal as evident from the fact that although killing of adult *S. mansoni* worms by treatment with schistosomicidal drugs has been accomplished in millions of people (many with a high worm burden), symptoms associated with dying worms have generally not been recorded. Only in a few cases worm death was associated with acute disease[8].

Schistosomes have a long life span suitable for a prolonged expression of transgenic gene products in vivo: *S. mansoni* can live for decades as it was found in Yemeni immigrants to Israel[49]. Their prolonged survival in the definitive host points to the existence of mechanisms of immune evasion[50]. Even when an immune response develops against these parasites it will affect the young worms (schistosomula) at various stages after penetration and migration within the definitive host, and even then, mechanisms of immune attrition can affect only a fraction of the re-infecting larvae[50]. In man, immunity against schistosomes can be detected during puberty after a prolonged infection[51,52], although partial age-dependent resistance may also exists[52,53]. Various humoral factors have been demonstrated to participate in human immunity to schistosomes[54] while the role of cellular mechanisms is as yet unclear. Also, various antigens have been proposed as protective antigens and are being studied as candidate antigens for vaccination[55]. The role of the eggs on the development of immunity and resistance against *S. mansoni* (the most studied schistosome species) is well documented in animal models[56,57], therefore infection by unisexual transgenic schistosomes will most likely not only avoid the major pathological feature of schistosomiasis as described above, but may also decrease attrition of the parasites and thus enable even longer survival than recorded for natural infection of man[52]. Schistosomes can therefore be considered as well adapted, long living universal transplants in their specific hosts, and transgenic schistosomes can be expected too to survive for a long time in their host.

Schistosomes do not multiply in their vertebrate host and they can be easily introduced into their hosts or removed from it, to enable control of the worm burden and hence of the quantity of transgene products: Cercariae of schistosomes penetrate actively through the skin of target host when it becomes exposed to waterborne cercariae by dipping[8,11]. Cercariae can alternatively be injected into the skin directly or following artificial transformation to schistosomula, which can even

be cryopreserved for long term storage[58]. Following penetration cercariae transform rapidly into schistosomula which migrate via the blood stream to the lungs and then to the liver where they mature, and subsequently migrate to their target location. Since the sex of the cercaria can be easily determined by the polymerase chain reaction (PCR)[59] the identification and selection of unisexual males, the transgenic transplant of choice, can be readily done. It was already mentioned that the worm burden can be determined by the size of the infective dose, and that replenishing is possible until a desired therapeutic effect is achieved. In addition, removal of schistosomes is possible by a number of new and effective drugs (notably praziquantel) which are being widely used for individual and community-based chemotherapy. Prazyquantel is very effective by a single oral dose (sometimes divided) and yields a high percentage of cure[60]. This should potentially enable removal of transgenic worms from treated individuals at any desired time. The monitoring of schistosome worm burden is possibly by examining circulating antigens in their host[61]. This should make possible control of schistosome worm burden.

Prevention of environmental dissemination of transgenic schistosomes can be expected to be straightforward: The capacity of schistosomes to be transmitted in nature depends on presence of specific infected snails shedding cercariae in sites where definitive hosts (humans or animals) contact the water. Egg-bearing excreta are the source of snail infection and therefore introducing unisexual transgenic cercariae into target hosts is not expected to result in environmental contamination with eggs.

In addition to schistosome species infecting man there are those which infect farm animals, thus the new technology should be suitable for animal experimentation for basic science purposes as well as for veterinary/animal husbandry purposes: The hosts of various schistosome species occurring in Africa are well known[62]. Among them *S. mansoni* and *S. haematobium* are essentially human parasites, but other species of African schistosomes can infect cattle, sheep, goat, and other ruminants of economic importance. In addition, *S. japonicum* which infects people in the Far East also infects 31 species of wild mammals and 13 species of domestic animals including dog, pig, cow, water buffalo and goat[8]. All species of schistosomes can be used for the proposed technology. GST genes of schistosomes other than *S.*

mansoni[63] have been identified[64], and may be used as optional sites for inserting desired genes. Also, highly repeated sequences similar to the one described to *S. mansoni*[28] are also present in the genome of other schistosome species including *Schistosoma haematobium* (see SEQ ID NO: 9, disclosing a *DraI* repeat sequence in the genome of *Schistosoma haematobium*, estimated to present in 50,000 copies per genome), and are potential sites of gene insertion. The new technology presented here for *in vivo* expression of desired gene product by the parasite, can expand the use of the mouse, and of other laboratory animals compatible for schistosome infection, for a variety of basic and applicable research purposes.

EXAMPLE 2

Materials and Methods

Parasite strain, maintenance of its life cycle and harvesting of parasites: *Schistosoma mansoni*, an Egyptian strain, was originally obtained from Wellcome Laboratories 35 years ago and its life cycle maintained by standard methods in outbred albino mice and in its snail host *Biomphalaria glabrata*. Briefly, livers were collected from infected mice about 9 weeks after subcutaneous injection with 350 cercariae. Numerous ova surrounded by granulomata were embedded in the liver tissue. The livers were homogenized and the eggs washed several times in 1.7 % NaCl solution (to avoid miracidial hatching under inadvertent hypotonic conditions). Distilled water was added to the final egg sediment and hatching of miracidia was enabled for 30 minutes. Miracidia were collected from the egg suspension in a dark room and under a narrow beam of light (which attracts these phototrophic larvae). Snails were exposed in separate small containers to 5-10 miracidia each for 16 hours under fluorescent light source. At least 24 snails were infected as a batch. Six or more weeks later, after completion of asexual multiplication of larvae within the snails, cercariae were shed from the snails under light in a beaker containing dechlorinated water, and 7 week old female mice were injected subcutaneoulsy with 350 cercariae. *S. mansoni* clean eggs for electroporation were harvested as previously described[65,66] from intestines of mice infected 9 weeks previously with wild type (WT) schistosomes. Adult worms were isolated from the mice 6-10 weeks later by liver perfusion[67].

Selection of electroporation conditions: Clean schistosome eggs were sedimented by centrifugation (1000 rpm/3 min) and washed once in 10 ml of cold RPMI1640 medium supplemented with geneticin and with 15 % fetal calf serum. Finally the eggs were resuspended in RPMI, and a 0.2 ml egg suspension (containing $2.5x10^3$ or $5x10^3$ eggs) transferred to an electroporation cuvette (0.4 cm wide) and exposed to various electroporation conditions as follows in a Gene Pulser (Bio-Rad) with exponential discharge: 500 V, 750 V, 100 V and 1500 V from a 25 μF capacitor; 1500 V and 2000 V from a 0.25 μF capacitor; 250 V and 450 V from a 125 μF capacitor and from a 500 μF capacitor. Electroporated eggs were left at room temperature for 20 minutes then 0.8 ml double distilled water was added for hypotonicity and the quvettes left for 0.5 hours under light, for enabling hatching of miracidia. Snails were exposed individually to 5-10 miracidia/snail for 16 hours under light. Hatching and viability (motility) were subsequently determined in search of electroporation conditions that will yield about 50 % viability of miracidia. For determining hatching rate, the content of each cuvette was transferred to a well of a 24 well culture plate, lugol was added for killing the miracidia and for staining, and about 100 ova and miracidia in the same area were screened. The number of free miracidia and of empty ova (eggshells) were determined and the average between them provided the hatching rate (% hatching). For determining viability by motility, the contents of each cuvette was centrifuged at 1000 rpm for 2 minutes to sediment eggs and dead miracidia and motile miracidia in the supernatant were collected, immobilized and stained with lugol, and counted. Control counts were carried out with eggs kept under similar conditions but without pulsing.

Based on the results obtained from preliminary experiments (see Results below) the following conditions were employed in the present work: Eggs (0.2 ml suspension containing $2.5x10^3$ or $5x10^3$ eggs) were mixed with plasmid DNA (5-10 mg/$5x10^3$ eggs) then transferred to an electroporation cuvette (0.4 cm wide) and exposed to the selected electroporation conditions. These were exponential discharge of 500 V (1250 V/cm) from a 25 μF capacitor. Electroporated eggs were left at room temperature for 20 minutes then 0.8 ml double distilled water was added for hypotonicity and the quvettes left for 0.5 hours under light, for enabling hatching of miracidia.

In other experiments free swimming miracidia were prepared by inducing eggs to hatch under similar conditions and transformation was carried out by electroporation under the same gene pulsing conditions described above for miracidia within the eggs. Two techniques were developed for this purpose, as follows.

Miracidia from schistosome eggs in liver of mice: Livers from mice infected 8 weeks previously were homogenized and washed as described above for maintenance of schistosome life cycle. Ova were left in deionized water for hatching in a dark room and cercariae attracted to a beam of light were collected. About 6 ml of suspended miracidia were collected in a conical centrifuge tube. One ml of diluted (1/5) normal saline was added and miracidia (n=80-160/400 µl) were distributed to electroporation cuvettes (0.4 cm wide). Construct DNA (5-10 µg/80-160 miracidia) was added to each cuvette separately just prior to electroporation under the selected electroporation conditions. These were exponential discharge of 500 V (1250V/cm) from a 25 µF capacitor. Under these conditions the discharge velocity was 5-6 milliseconds (ms).

Miracidia from eggs in intestines of infected mice. Harvesting of eggs from intestines yielded many more eggs (*ca.* 500,000/10 mice) than from livers. Following harvesting of eggs and washing with RPMI as described above, 7 ml of phosphate buffered saline diluted 1/6 was added. The egg were then distributed in a small Petri dish (3 cm diameter), and placed under light for hatching for 45 minutes. Miracidia (80-120/400 µl) were placed in a cuvette and electroporated as described above.

Following the development of the parasites after gene pulsing: Snails were exposed individually to 5-10 miracidia/snail for 16 hours under light. Cercariae were shed beginning from 6 weeks later as described above (see section on maintenance of life cycle). Development of cercariae was followed by periodic (weekly to triweekly) determination of numbers cercariae shed from snails. In initial experiments only approximation was employed by grading cercarial shedding from "a few cercariae" (+) through "very strong shedding" (++++). These grades were given numerical values as follows: 10 (+), 100 (++), 500 (+++) and 1500 (++++). In subsequent experiments numbers of cercariae/snail were determined. Cercariae harvested from snails infected with transformed miracidia underwent analysis confocal

microscopy (see below) or were employed to infect mice, which in turn were a source of adult worms, collected 7-10 weeks later by perfusion[67].

Isolation of cercarial genomic DNA: Cercariae were shed from infected snails infected by wild type (WT) or electroporated miracidia from the sixth week after exposure to miracidia. Genomic DNA was isolated from cercaria by lysing cells in 0.1 % Triton x 100 + TEN (10 mM Tris-HCl pH 8.0, 10 mM EDTA and 10 mM NaCl). The lysate was treated with RNase A (0.1 mg/ml) in 37 °C for 30 minutes and then with Proteinase K (0.1 mg/ml) in 37 °C for 30 minutes. Residual proteins were removed from the lysate by phenol extraction followed by phenol-chloroform extraction. Genomic DNA was recovered by ethanol precipitation.

Analysis of genomic DNA: Aliquots of *Schistosoma mansoni* cercarial DNA (>1 ng) underwent amplification by the polymerase chain reaction (PCR) with primers specific for the GFP gene at an internal position (see paragraph on primer design and PCR conditions, below). Products of the PCR were separated by agarose gel electrophoresis and stained with ethidium bromide.

Plasmids construction: All the plasmids employed were derived from the commercially available pBluescript plasmids (Stratagene). Into the *Eco*RV restriction enzyme site of these plasmids introduced was a 1900 bp long fragment from the 5' end of genomic copy of the GST gene[23] isolated from genomic DNA of *Schistosoma mansoni* by PCR reaction using specific primers GST-1 and GST-2 (see paragraph on primers design and PCR conditions, below). Figure 1 provides a schematic representation of the GST gene.

The GFP gene which encodes the green fluorescence protein from the medusa *Aequorea victoria*[33,34] was than inserted into the PstI site located in the second exon of the GST gene[23] ("GST-GFP fusion gene"). The GFP coding sequence that was used, was obtained from plasmid pGFP by PCR reaction with specific primers covering most of the entire gene (see paragraph on primer design, below). The size of the GFP fragment was designed to fit the reading frame of the GST protein in exon 2. The resulting construct contains a fusion of the GST-GFP proteins in the same reading frame. This construct is expected to produce the fluorescent protein under the regulation of gene expression of GST from *Schistosoma*. This fusion protein also contains the peptide sequences that are targeting this protein to cellular compartments were

the native GST protein resides, namely passage through the tegument of the parasite. Due to the long homology between the plasmid harboring the GFP and the GST genomic sequences, the cassette of GFP-GST can be incorporated by homologous recombination and stably expressed as part of the genome. Alternatively, GFP protein with the GST leader and under regulation of the GST promoter sequence may be transiently expressed by the vector positioned as an episome. The GFP was also introduced inversely for obtaining a control construct in which expression is not expected. Figure 2 provides a schematic representation of the GST-GFP fusion gene.

In order to achieve better incorporation of the expression vectors into the Schistosomal genome the long homology of the GST gene was replaced with shorter homology of the sequence SM1-7. This sequence is found in the genome in high copy numbers (10 %)[28] therefore the vector may target many sites in the genome. The regulatory sequences that were used in this type of vector included the promoter of GST. For construction of this vector the major part of the 5' end of the GST sequence was deleted by digestion with *Cla*1I and *Afl*III restriction enzymes, as well as all of the 3' end of this gene in the GST-GFP fusion vector, leaving only the promoter region (the first 200 nucleotides of the GST gene). Five copies of SM1-7 sequence were introduced into the *Xho*I and *Xba*I restriction enzyme sites flanking the GST promoter of the GFP gene fusion. Figure 3 provides a schematic representation of this construct.

Primer design and PCR conditions: Two sets of primers were used in plasmids construction, the GST primers were used for amplification of the GST gene from the genome of *Schistosoma* and the second set of primers was used to amplify the GFP coding region from plasmid pGFP. The computer program Oligo4 was used to select the optimal and unique primers for both genes.

Primers to amplify the GST gene were: GST-1: 5'-CATCGAGA ACGGTTTACATGTTCA-3' (SEQ ID NO:3); GST-2: 5'- GCA GCCTCCTCACATGCTCCA-3' (SEQ ID NO:4)

The PCR for amplification of the GST gene was performed under the following conditions: 30 cycles of: denaturation - 94 ^{o}C, 60 sec; annealing - 55 ^{o}C, 60 sec; and elongation - 72 ^{o}C, 3 min.

Primers to amplify the GFP gene were: uGFP: 5'-ATGAGTAAAGGAGAAGAACTTTTC-3' (SEQ ID NO:5); and lGFP: 5'- TTTGTATAGTTCATCCATGCC-3' (SEQ ID NO:6).

A pair of internal primers specific for the GFP gene were designed for detection of GFP in transfected Cercariae by PCR. These were as follows: GFP1: 5'-TCTCCCATGATGTATACATTATGT-3' (SEQ ID NO:7); and GFP2: 5'-TCTCCATCGAAGGGTCATCACG-3' (SEQ ID NO:8).

The PCR for amplification of the GFP gene and fragments was performed under the following conditions: 25 cycles of: denaturation - 94 ^{o}C, 30 sec; annealing - 51 ^{o}C., 30 sec; and elongation - 72 ^{o}C 60 sec.

Sequencing: The sequences of the constructs developed were confirmed by double stranded sequencing carried out automatically by the Dye Deoxy Sequencer (Applied Biosystems). The sequencing results are shown in Figures 4 and 6 (SEQ ID NOs: 1 and 2, respectively).

Examination of cercariae and adult worms by confocal microscopy: Cercariae shed from snails infected with WT miracidia or with miracidia that underwent electroporation with recombinant plasmids were analyzed directly after they were shed, or following fixation with 3 % paraformaldehyde, treatment with 50 mM NH_4Cl, and washing with PBS. Adult worms were collected from infected mice and examined directly or following fixation. Scanning laser confocal microscope (Zeiss 410) was employed to examine cercariae and adults by laser excitation at 488 nm, employing an excitation filter FT 510 and an emission filter LT 515. Contrast level used was 310-320.

Examination of cercariae and adult worms by anti-GFP: This examination was to confirm the presence of GFP in the transfected parasites. The indirect fluorescent antibody technique (IFAT) was employed for this purpose with anti-GFP monoclonal antibody (Clontech, USA) as a first antibody, and Cy5-labeled goat anti-mouse IgG (Chemicon, USA), as a second antibody. Parasites were incubated at room temperature for 0.5 hours (cercariae) or one hour (adult worms) with 3 % formaldehyde in PBS, then washed once with PBS and incubated for 0.5 hours at room temperature in 50 mM NH_4Cl for blocking free aldehyde groups, and washed again with PBS. Permeabilization was carried out by incubation at room temperature for 0.5 hours with a solution containing 0.1 % triton X-100 and 1 % BSA in PBS, then washing with PBS. Blocking was carried out by incubation for

0.5 hours at room temperature in 5 % Goat serum. The first antibody (diluted 1:500) was reacted with the parasites at 37 °C for 0.5 hours or overnight at 4 °C and following four washes in PBS the second antibody (diluted 1:400) was reacted with the parasites for 0.5 hours at 37 °C. The parasites were examined by confocal microscopy after mounting in a solution containing 86 % glycerol, 10 % PBS, 0.1 % NaN_3, and 3-4 % DABCO (1,4 Diaza Bicyclo (2.2.2) Octane. The scanning laser confocal microscope (Zeiss 410) was employed with excitation by a Helium-Neon Laser at 650 nm, and emission at 680 nm.

EXAMPLE 3

Experimental Results

Sequence of our recombinant plasmids: Figure 4 presents the sequence of the vector carrying the GFP-GST fusion coding information (SEQ ID NO:1). Figure 5 presents the map of this recombinant plasmid. Figure 6 presents the sequence of the vector in which the GFP was inserted within the SM1-7 repeated sequence (SEQ ID NO:2). Figure 4 presents the map of this recombinant plasmid

Electroporation conditions: Percent hatching of miracidia from ova which underwent electroporation was higher than in control unpulsed ova in 6 out of 10 electroporation conditions tested. Percent hatching was similar to control in 2 electroporation conditions (25 μF/1500 V and 0.25 μF/ 2000V), and lower than control values in 2 other electroporation conditions (25 μF/ 500V, and 0.25 μF/1500 V). The results of one experiment (out of two) are presented in the Table 1 below.

TABLE 1

PULSE:	0	25 μF---------->			0.25 μF--------->		125 μF------->		500 μF-------->		
		500 V	750 V	1000 V	1500 V	1500 V	2000 V	250 V	450 V	250 V	500 V
% HATCH:	20	18.8	28	23.4	20.5	14.4	20.2	29.7	25.4	29.4	26.6

Percent motility of miracidia hatched from ova which underwent electroporation was invariably lower than that obtained with control unpulsed ova. The results of one experiment are presented in Table 2 below.

TABLE 2

PULSE: 0	25 μF----->			0.25 μF----->		125 μF----->		500μF----->		
	500 V	750 V	1000 V	1500 V	1500 V	2000 V	250 V	450 V	250 V	500V
% MOTILE: 46	20*	8	7	2	45	40	34	23**	12	8

* Selected conditions; **Immediate alternative option.

In three subsequent experiments where cercarial viability (motility) was measured, the selected electroporation conditions (25 μ F/500 V) were employed for introducing] foreign DNA (5-10 mg/cuvette). The count of motile miracidia was quarter to half of the control.

Rate of cercariae development: Cercariae were shed in a pool of snails (at least 10 snails/pool but usually more at the time of shedding). Cercarial shedding in normal schistosome/snail combination typically starts 4 weeks after exposure to 5-10 miracidia, it usually reaches a maximal rate ranging between 1000 and 2800 cercariae/snail, and the peaks of cercarial shedding is between days 60 and 90 following exposure. In two control experiments where electroporation was done without the presence of DNA, cercarial shedding reached normal high values, but the peak was somewhat delayed (between 80 and 110 days following exposure) and remained high until the last measurement taken (124 days following exposure). In 4 out of 8 transformation experiments with the GFP-GST fused construct, cercarial counts were very low with "tens" of cercariae/snail or less recovered throughout the monitoring period (up to 200 days after the snails were exposed to miracidia). In two out of these 8 experiments very strong shedding started only 120 days, or 160 days after exposure to miracidia. In another two out of these 8 experiment cercarial shedding peaked early (at about 60 days after exposure) and reached over 2000 cercariae/snail for 2-3 weeks, then declined rapidly. In one of these two later experiments electroporation conditions were milder (25 μF/400V instead of 25 μF/500 V). In 2 out of 4 experiments in which the GFP-SM1-7 construct was employed for transformation, cercarial shedding was with multiple peaks reaching several hundred cercariae/snail (range of 500 to 800). In one of these 4 experiment a single low peak (about 200 cercariae/snail) was exhibited at about 100 days post exposure. In one of these 4 experiments altered electroporation conditions were employed (25 μF/400 V instead of 25 μ

F/500 V). High infection was obtained from the very early cercarial shedding (42 days) and fluctuated between 1200 cercaria/snail and about 2700 cercariae/snail. Table 3 below summarizes the results of cercarial counts.

TABLE 3

Miracidia used to infect snails	Maximum No. cercariae/snail	Time of high cercariae no./snail (>1000)	Duration of high shedding
Wild type	1-2.5 x10^3	day 60 to 90 PE[a]	60 days
Pulsed W/O DNA	+ + + +	day 80-130 (estimate)	50 days (estimate)
Pulsing control	+ + + +	day 80-130 (estimate)	50 days (estimate)
"GFP in GST"			
Exp. # 3:	+ + + +	day 164 to 193 PE[a]	Min. 26 days
Exp. # 5:	+ + + +	day 123 to 142 PE[a]	Min[b]. 21 days
Exp. # 6:	+ + (one + + +)	Practically irrelevant	
Exp. # 7:	+ + (one + + +)	Practically irrelevant	
Exp. # 8:	+ +	Practically irrelevant	
Exp. # 9:	+ +	Practically irrelevant	
Exp. # 15:	2500	day 47-118 PE[a]	Min[b]. 71 days
Exp. # 16[c]:	2000	day 49-106 PE[a]	Min[b]. 54 days
"GFP in Sm1-7"			
Exp. # 13:	+ + + (under 1000)	day 55 to 146 PEa	
Exp. # 14:	+ +	Practically irrelevant	
Exp. # 15:	+ + (a single + + +)	Practically irrelevant	
Exp. # 16[b]:	2750	day 43 to 106 PEa	Min[b]. 73 days

Relative values for cercarial shedding: marginal (+), weak (+ +), medium (+ + +), strong(+ + + +). a = Post Exposure; b = Minimum. c = reduced voltage (see text).

The results on development of cercariae following infection of snails by transformed miracidia are presented in Figure 14. Five transfection experiments are presented. The development of cercariae from snails infected by transformed miracidia (exposed to electroporation with the GST-GFP construct) is very similar to development of snails infected by wild type cercaria without electroporation.

It can be concluded for practical purposes that although introducing foreign genes may cause sometimes a reduction in the number of shed cercariae or a delay in cercarial shedding this does not interfere with the practical usefulness of transgenic cercariae since sufficient numbers are obtained for infecting the definitive host. The trend with cercariae to which GFP-SM1-7 construct was introduced, was towards a more efficient shedding. It therefore ensues that ongoing monitoring of cercarial shedding is required with several replicate

experiments, and a clone exhibiting desired qualities may then be selected for propagation by transplantation of sporocysts to normal snails and for infecting the vertebrate host.

Rate of development of adult worms: In two experiments where mice were exposed to cercariae from experimental snails to which the GST-GFP construct was introduced as made evident by PCR (70-75 cercariae/mouse for active skin penetration) no adult worms were recovered. When wild type cercariae are used for the active penetration method, the number of adult worms recovered is about 25 % of the number of cercariae to which mice were exposed. In other experiments, cercariae were injected subcutaneously. When wild type cercariae are used for infecting mice by injection, the number of adult worms recovered fluctuates between 1 % and 10 % of the number of cercariae introduced. The results of these experiments with transgenic cercariae were as summarized in table 4 below.

TABLE 4

Identity of cercariae injected	No. of recovered adult worms/mouse
GST-GFP transgenic	3, 0, 30, 3, 21
GST-GFP (inverse) transgenic	27, 10, 0
Sm-7-GFP transgenic	5, 20, 18, 17, 33, 42, 52
Wild Type	5, 30, 5, 25

In subsequent experiments where transformation was done on free swimming miracidia, cercariae were injected subcutaneously into mice (400 cercariae/mouse) and adult worms were recovered 6-9 weeks later the recovery of adult worms was as follows:

TABLE 5

Identity of cercariae injected worms/mouse	No. of recovered adults
GST-GFP transgenic	66, 32, 19
Sm-GFP transgenic	179, 78

The trend with cercariae transformed with the Sm-GFP construct was towards a more efficient development into adults.

Incorporation of GFP gene into the schistosomal genome: Figure 8 presents a representative PCR experiment demonstrating amplification of the GFP region by corresponding primers, when PCR was carried out with total genomic DNA prepared from cercariae. Amplification signals were demonstrated whether the GSP sequence was introduced directly (B), or inversely (A) within the schistosomal genome. Introduction of the transgenes into the schistosomal genome was thus demonstrated. However, simultaneous presence of the recombinant vector as an episome was not excluded. Demonstration by PCR of the incorporation of the GFP-SM1-7 transgene into the schistosomal genome is still required (although expression was clearly demonstrated by confocal microscopy as described below).

In further experiments with cercariae obtained from snails infected by miracidia which underwent electroporation as free swimming organisms, PCR results were as presented in Figure 15. Positive reactions were obtained in several transformation experiments with GST-GFP constructs both in direct (expressing) and inverse positions. Positive results were also obtained in transformation experiments with the Sm-GFP construct.

Cercariae proven transgenic were used to infect mice and adult worms developing from them underwent DNA extraction and PCR for detecting insertion of the GFP gene. Figure 16 presents the results of several such experiments. Positive amplification signals were obtained in experiments where transfection was done with all the constructs used, namely GST-GFP in both the direct and inverse position, and also with the Sm-GFP construct. This thus completes the demonstration of the stable transfection of schistosomes by group transformation methods and the maintenance of stable integration throughout the schistosome developmental stages from miracidium to adult worms. The adult worms which develop in the definitive host are the carrier of the transgene which is integrated in their genome and are capable of its expressing (as is further shown below). Interestingly, the various genes were found in most cases only in adult male worms. Only occasionally transgenic females were found (this is demonstrated by confocal microscopy below).

Expression of GFP in transgenic schistosomes:

a. Cercariae: GFP was expressed by most of the cercariae examined (1-10/group depending on availability) in some experiments

only part of the cercariae (20-60 %) were positive. Although a statistical analysis was not done due to the small number of cercariae examined in each experimental group, these results suggest a very efficient transgenesis. When more than one miracidium is used for infecting a snail a mixture of positive and negative cercariae is not surprising considering that not all of the miracidia became effectively transfected. Positive signals were diffuse or concentrated in discrete foci. Representative results of confocal microscopy with cercariae are presented in Figures 9a-d and 10a-d. Figures 9a-d demonstrates a gallery of 4 photographs of experiments where the GFP-GST construct was employed. GFP-positive cercariae are demonstrated in Figures 9a-c, whereas Figure 9d is of a group of wild type cercariae. In Figures 9a-b fluorescence (yellow-white) is seen over a red background. In Figure 9c fluorescence (red) is seen in combination with Nomarski microscopy. Figure 9d shows several cercariae with negative signal (only the red background is visible). Figures 10a-d present a gallery of photographs, three (Figures 10a-c) with positive signals from experiments where the GFP-Sm1-7 construct was introduced, and one (Figure 10d) wild type as control. Please note the pronounced fluorescence observable in the experimental images (Figures 10a-c) as compared with control (Figure 10d).

b. Adult worms: Figures 11a-i present a gallery of nine photographs as follows: Figures 11a-d show a worm from an experiment in which the GST-GFP (inverse) was employed, Figure 11e shows a wild type worm, and Figures 11f-i show a worm from an experiment in which the GST-GFP was employed. Strong expression typified the male worm in Figures 11f-i. Focal fluorescence (autofluorescence) at the tips (mouth and tail) of the male worm of Figures 11a-d was also observed. Focal fluorescence at the anterior was also present in the wild type worm of Figure 11e. Figures 12a-f present a gallery of six photographs of adult worms, in which Figure 12a shows a strongly positive male-female pair from a GFP-GST transgenesis, Figures 12a-b show two positive male worms from an GFP-Sm1-7 transgenesis experiment, Figures 12d-e show two negative male-female wild type worms, and Figure 12f show a negative female wild type worm showing non specific fluorescence of the ovarium (non specific fluorescence of the gut is also found when pigment resulting from digestion of hemoglobin is present).

Anti-GFP tests: Figures 13a-d present a gallery of four photographs taken with transgenic cercariae which underwent fluorescent antibody staining. The photographs of Figures 13a-b were taken with bodies of GFP-GST transgenic cercariae, one of them (13b) with fluorescent Cy-5 labeled antibody staining (red over blue) staining, and the other with both GFP (green) and Cy-5 fluorescent antibody (red) staining. The other 2 cercariae shown in Figures 13c-d were from an experiment with GFP-SM1-7 transgenesis. They were viewed by both GFP and CY-5 staining. Differences in intensity can be observed. Examining adult worms is particularly important because of the non specific fluorescence observed in some cases at the anterior and posterior ends and at the position of the ovarium.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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Additional references are cited within the text. Citation or identification of any reference in any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention. In addition, all publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A eukaryotic diploid multicellular parasite transformed with a transgene.

2. The parasite of claim 1, wherein the parasite is a worm.

3. The parasite of claim 2, wherein said worm is a flat worm.

4. The parasite of claim 3, wherein said flat worm is a trematode.

5. The parasite of claim 4, wherein said trematode is a schistosome.

6. The parasite of claim 1, wherein the parasite is infective to human or animal.

7. The parasite of claim 1, wherein the parasite is infective to human.

8. The parasite of claim 5, wherein said schistosome is infective to human.

9. The parasite of claim 5, wherein said schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis*.

10. The parasite of claim 1, wherein the parasite is sterile.

11. The parasite of claim 1, wherein the parasite is sensitive to a known drug.

12. The parasite of claim 1, wherein said transgene is integrated in the parasite genome.

13. The parasite of claim 1, wherein said transgene is integrated in the parasite genome.

14. The parasite of claim 1, wherein said transgene is integrated in a selected genomic locus in the parasite genome.

15. The parasite of claim 14, wherein said selected genomic locus is a repetitive sequence.

16. The parasite of claim 14, wherein said selected genomic locus is a unique sequence.

17. The parasite of claim 1, wherein the parasite has distinguishable sexes.

18. A method of providing a eukaryotic host with a protein or polypeptide, the method comprising the step of infecting the eukaryotic host with a eukaryotic diploid parasite transformed with a polynucleotide sequence encoding the protein or polypeptide.

19. The method of claim 18, wherein said protein or polypeptide is secreted from said parasite.

20. The method of claim 18, wherein said infection is by a plurality of individuals of said parasite, all of said individuals are of a single sex.

21. The method of claim 20, wherein said single sex is selected from the group consisting of male and female.

22. The method of claim 18, wherein said parasite is a worm.

23. The method of claim 22, wherein said worm is a flat worm.

24. The method of claim 23, wherein said flat worm is a trematode.

25. The method of claim 24, wherein said trematode is a schistosome.

26. The method of claim 18, wherein said host is human or animal and said parasite is infective to said human or animal.

27. The method of claim 18, wherein said host is human and said parasite is infective to human.

28. The method of claim 25, wherein said schistosome is infective to human.

29. The method of claim 25, wherein said schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis*.

30. The method of claim 18, wherein said parasite is sterile.

31. The method of claim 18, wherein said parasite is sensitive to a known drug, said drug is therefore effective in removing said parasite from said host.

32. The method of claim 18, wherein said polynucleotide sequence is integrated in said parasite's genome.

33. The method of claim 32, wherein said integration is by homologous recombination into a selected genomic locus.

34. The method of claim 33, wherein said selected genomic locus is a repetitive sequence.

35. The method of claim 33, wherein said selected genomic locus is a unique sequence.

36. The method of claim 18, wherein said parasite has distinguishable sexes, whereby a single sex of said sexes is used for said infection.

37. A method of genetically modifying a eukaryotic diploid parasite, the method comprising the step of transforming the eukaryotic diploid parasite using a group transformation method.

38. The method of claim 37, wherein the parasite is a worm.

39. The method of claim 38, wherein said worm is a flat worm.

40. The method of claim 39, wherein said flat worm is a trematode.

41. The method of claim 40, wherein said trematode is a schistosome.

42. The method of claim 37, wherein the parasite is infective to human or animal.

43. The method of claim 37, wherein the parasite is infective to human.

44. The method of claim 41, wherein said schistosome is infective to human.

45. The method of claim 41, wherein said schistosome is selected from the group consisting of *Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum, Schistosoma bovis, Schistosoma mattheei, Schistosoma rhodhaini, Schistosoma magrebowiei, Schistosoma intercalatum, Schistosoma curasoni, Schistosoma mekongi, Schistosoma spindale, Schistosoma leipere, Schistosoma turkestanicum, Schistosoma inidicum, Schistosoma nasalis and Schistosoma suis.*

46. The method of claim 37, wherein the parasite is sensitive to a known drug.

47. The method of claim 37, wherein said group transformation method is selected from the group consisting of electroporation, chemical transformation, lipofection and biolistic bombardment.

48. The method of claim 37, wherein said transgene is integrated in the parasite genome.

49. The method of claim 37, wherein said transgene is integrated in a selected genomic locus in the parasite genome.

50. The method of claim 49, wherein said selected genomic locus is a repetitive sequence.

51. The method of claim 49, wherein said selected genomic locus is a unique sequence.

52. The method of claim 37, wherein the parasite has distinguishable sexes.

Fig. 1

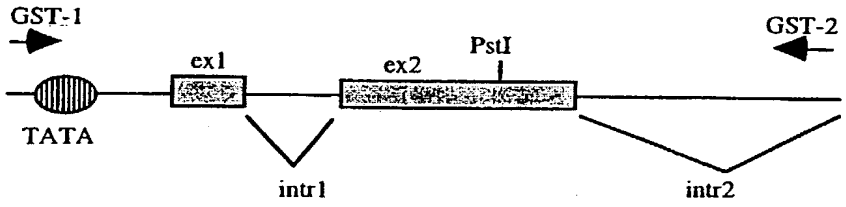


Fig. 2

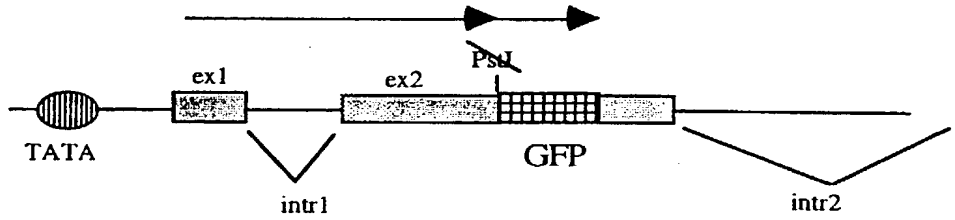


Fig. 3

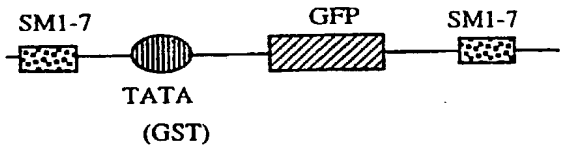


Fig. 4

GSTGFP Length: 5356 February 18, 1998 09:24 Type: N Check: 5900 ..

features:

GFP gene: 56-769
the first part of the genomic GST gene: 4460-5350
the second part of the genomic GST gene: 769-1600
the Amp resistance gene (complementary): 3642-2809

```
   1  AGCTTGCATG CCTGCAGGTC GACTCTAGAG GATCCCCGGG TACCGGTAGA
  51  AAAAATGAGT AAAGGAGAAG AACTTTTCAC TGGAGTTGTC CCAATTCTTG
 101  TTGAATTAGA TGGTGATGTT AATGGGCACA AATTTTCTGT CAGTGGAGAG
 151  GGTGAAGGTG ATGCAACATA CGGAAAACTT ACCCTTAAAT TTATTTGCAC
 201  TACTGGAAAA CTACCTGTTC CATGGCCAAC ACTTGTCACT ACTTTCTCTT
 251  ATGGTGTTCA ATGCTTTTCA AGATACCCAG ATCATATGAA ACGGCATGAC
 301  TTTTTCAAGA GTGCCATGCC CGAAGGTTAT GTACAGGAAA GAACTATATT
 351  TTTCAAAGAT GACGGGAACT ACAAGACACG TGCTGAAGTC AAGTTTGAAG
 401  GTGATACCCT TGTTAATAGA ATCGAGTTAA AAGGTATTGA TTTTAAAGAA
 451  GATGGAAACA TTCTTGGACA CAAATTGGAA TACAACTATA ACTCACACAA
 501  TGTATACATC ATGGCAGACA AACAAAAGAA TGGAATCAAA GTTAACTTCA
 551  AAATTAGACA CAACATTGAA GATGGAAGCG TTCAACTAGC AGACCATTAT
 601  CAACAAAATA CTCCAATTGG CGATGGCCCT GTCCTTTTAC CAGACAACCA
 651  TTACCTGTCC ACACAATCTG CCCTTTCGAA AGATCCCAAC GAAAAGAGAG
 701  ACCACATGGT CCTTCTTGAG TTTGTAACAG CTGCTGGGAT TACACATGGC
 751  ATGGATGAAC TATACAAATA GCATTCGTAG AATTCTATTG CACGGTATAT
 801  GGCGAAGAAA CATCATATGA TGGGTGAAAC AGACGAGGAA TACTATAGTG
 851  TTGAAAAGTT GATTGGTCAG GTGAGTTGAG CCTTTTATTG GTTGATGGGA
```

Fig. 4 (continued)

```
 901  TTTTATGCTA CTGGAGTCCT GTAAGTTCAG TTTGTTGCGT TGAATAAATA
 951  AAGTTCGAAT TGATAGGCGA GTGGTAACCT ATGTGTTGTG ATTACTAAAA
1001  GTCATAAGTG TGAGGTATCC CAATTATTAG AAATGTGACT AAGACTGAGC
1051  TTTTCTGCCT ATTGGGATAT TACAGATAAG TTACTTATTT GTAGTATCGT
1101  AATAGTGTTC ACTGGGAACC ATCTAATTCA CTCTCAAAAA TACTTTATCG
1151  TGTCTTATTT TATTCATCCT CATAACTCGT TTGCTAGGAG AACAGCTGTG
1201  CCCATAGGTT AGTTGACAAG TTACTTACTT CCCCATGATA CGCCTTATTT
1251  TAATAACCTA ATAAAGGCTC GGTTACGTAA CGTCCCTATA GTGCATAGCA
1301  TACCTACGAA CATTGACTCC CAACTGATTA AGTGCGGATT GCATTTTTTT
1351  GGATGTTATC GCACAGTAAG ACAATACCAT CCTCATCTCA ATGTCAAGAG
1401  GTCTTTCTCC AGGCAACAGA TCCATACCAC CATTACTTAC ATCCATCAGA
1451  GCTGTTCCCA GAATGTCTTG TTATAAACGG ATGGATTATT TATTTATTTG
1501  ACCACATAAA TATTGCATCA AAGAGGTGGG GGATCCACTA GTTCTAGAGC
1551  GGCCGCCACC GCGGTGGAGC TCCAGCTTTT GTTCCCTTTA GTGAGGGTTA
1601  ATTGCGCGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG
1651  TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA
1701  AAGCCTGGGG TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC
1751  TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG
1801  AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG
1851  CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG
1901  GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA
1951  TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC
2001  GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC
```

Fig. 4 (continued)

2051 GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCGACAGGA

2101 CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC

2151 TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG

2201 GAAGCGTGGC GCTTTCTCAT AGCTCACGCT GTAGGTATCT CAGTTCGGTG

2251 TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGACCCCC CGTTCAGCCC

2301 GACCGCTGCG CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG

2351 ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG

2401 CGAGGTATGT AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC

2451 GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT

2501 TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG

2551 CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA

2601 AAAGGATCTC AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA

2651 GTGGAACGAA AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA

2701 GGATCTTCAC CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC

2751 TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG

2801 TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT

2851 GACTCCCCGT CGTGTAGATA CTACGATACG GGAGGGCTTA CCATCTGGCC

2901 CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA

2951 TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC

3001 AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG

3051 TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTACA

3101 GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG

3151 TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG

Fig. 4 (continued)

3201 CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA GTTGGCCGCA

3251 GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT

3301 GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT

3351 TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAATA

3401 CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG

3451 AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT

3501 CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC AGCATCTTTT

3551 ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC

3601 AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC

3651 TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA

3701 TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC

3751 ATTTCCCCGA AAAGTGCCAC CTGACGCGCC CTGTAGCGGC GCATTAAGCG

3801 CGGCGGGTGT GGTGGTTACG CGCAGCGTGA CCGCTACACT TGCCAGCGCC

3851 CTAGCGCCCG CTCCTTTCGC TTTCTTCCCT TCCTTTCTCG CCACGTTCGC

3901 CGGCTTTCCC CGTCAAGCTC TAAATCGGGG GCTCCCTTTA GGGTTCCGAT

3951 TTAGTGCTTT ACGGCACCTC GACCCCAAAA AACTTGATTA GGGTGATGGT

4001 TCACGTAGTG GGCCATCGCC CTGATAGACG GTTTTTCGCC CTTTGACGTT

4051 GGAGTCCACG TTCTTTAATA GTGGACTCTT GTTCCAAACT GGAACAACAC

4101 TCAACCCTAT CTCGGTCTAT TCTTTTGATT TATAAGGGAT TTTGCCGATT

4151 TCGGCCTATT GGTTAAAAAA TGAGCTGATT TAACAAAAAT TTAACGCGAA

4201 TTTTAACAAA ATATTAACGC TTACAATTTC CATTCGCCAT TCAGGCTGCG

4251 CAACTGTTGG GAAGGGCGAT CGGTGCGGGC CTCTTCGCTA TTACGCCAGC

4301 TGGCGAAAGG GGGATGTGCT GCAAGGCGAT TAAGTTGGGT AACGCCAGGG

Fig. 4 (continued)

4351 TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGAGC GCGCGTAATA

4401 CGACTCACTA TAGGGCGAAT TGGGTACCGG GCCCCCCCTC GAGGTCGACG

4451 GTATCGATAA GCTTGATTCA TCGAGAACGG TTTACATGTT CAATGAATCG

4501 AGTCAAATTT GTCTGCTTAA TTTTTATTGG TCACTCTTTC ACAGCCAATG

4551 AGGCACTCAA TAAACAGCGA ATAGAAATGA AATATTTACA GTTAAAATCA

4601 AGAGTTACAC TATTGGCCGA TCTGTTTACT AAATGACTAT TTAATAGTCA

4651 CAGCTCAGAT AGTTAGCACT GTTTTGTCTA TTTGCAAGAT GGCTGGCGAG

4701 CATATCAAGG TGGGTTGGTG TGGTTGTTCG TCATTGTGTT GCTTGGTAAA

4751 TTTTCGAGTG TTGTTAAGGT TCCTAATTGC AGCATTTAGC GGAATAAATA

4801 TTATTCCGCT ATATGAAGGT AAATACTGAG TCCATGTTAA TTGCACATTA

4851 GATCCGTACA TATATTTGGC TGTGATTTTC CACGTCATCA GGTCAGTAAG

4901 TTACGTGTTC ACACTGGGTT CTGATATAAT GCACAACCAT TATAGTCACA

4951 TAGTGGTCAC CGATTATGTG TTAGGTGTAG CATTTCAGGC GATGTTATAG

5001 TTTGTCTGTG GAATTGTTAC GAGAACTTAG GGGATTCTAG AGCGTGAATC

5051 CATCTAGTCC ATATCTCAGA GACATGCGTC CATGGAACAG TTATTTATTA

5101 TGTGATGTTT TGTCTGAAGT GGTTTTTTTC AAGCGTAAAT TGATGTGAAA

5151 CACAGCTGTA ATTTCTATAG GTTATCTATT TTGACGGACG CGGACGTGCT

5201 GAATCGATTC GGATGACTCT TGTGGCAGCT GGTGTAGACT ACGAAGATGA

5251 GAGAATTAGT TTCCAAGATT GGCCAAAAAT CAAACCAACT ATTCCAGGCG

5301 GACGATTGCC TGCAGTGAAA GTCACTGATG ATCATGGGCA CGTGAAATGG

5351 ATGTTA

Fig. 5

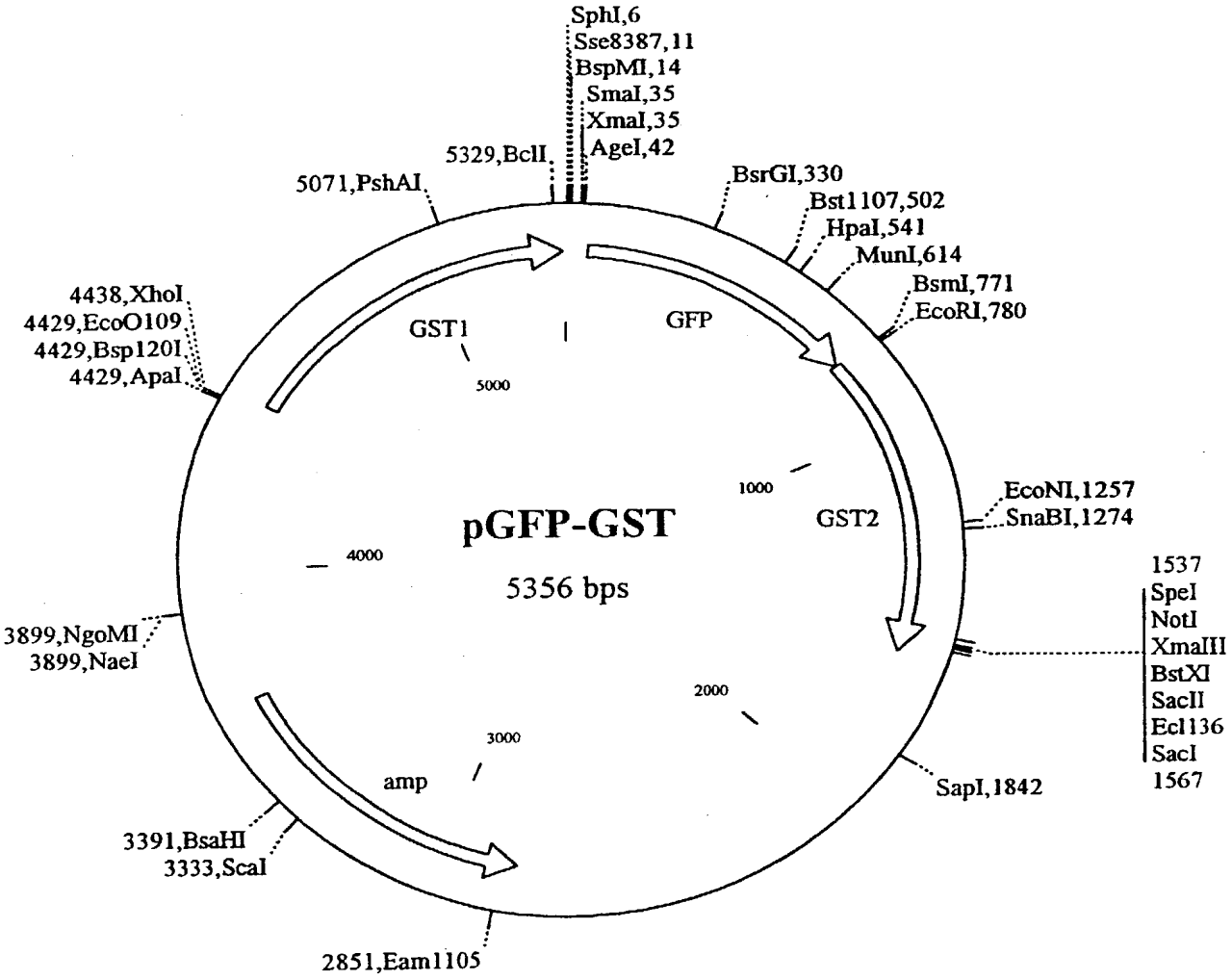

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GST2
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1537
SpeI
NotI
XmaIII
BstXI
SacII
Ecl136
SacI
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3899,NaeI
2000
3000
amp
SapI,1842
3391,BsaHI
3333,ScaI
2851,Eam1105

Fig. 6

pSm1-7 GFP SEQUENCE

Length: 6494 February 26, 1998 11:50 Type: N Check: 8163

Features:

first sm repeat	0-560
GST1 gene	560-1520
GFP gene	1530-2265
GST2 gene	2270-3022
second sm repeat	3025-3585

```
   1  GATCTGAATC CGACCAACCG TTCTATGAAA ATCGTTGTAT CTCCGAAACC
  51  ACTGGACGGA TTTTTATGAT GTTTGTTTTA GATTATTTGC GAGAGCGTGG
 101  GCGTTAATAT AAAACAAGAA TGATCTGAAT CCGACCAACC GTTCTATGAA
 151  AATCGTTGTA TCTCCGAAAC CACTGGACGG ATTTTTATGA TGTTTGTTTT
 201  AGATTATTTG CGAGAGCGTG GGCGTTAATA TAAAACAAGA ATGATCTGAA
 251  TCCGACCAAC CGTTCTATGA AAATCGTTGT ATCTCCGAAA CCACTGGACG
 301  GATTTTTATG ATGTTTGTTT TAGATTATTT GCGAGAGCGT GGGCGTTAAT
 351  ATAAAACAAG AATCATCTCA ATCCCATCAG CCGTTCTATG AAAATCGTTG
 401  TATCTCCGAA ACCACTGGAC GGATTTTTAT GATGTTTGTT TTAGATTATT
 451  TGCGAGAGCG TGGGCGTTAA TATAAAACAA GAATGATCTG AACACGGGTG
 501  TTTTTCTGTT CAGCTTATGC AACTTTAAAA TTCGATGGGT CGTCTCAACG
 551  AAATTTGTAT TGCTTTGTCG AGGTCGACGG TATCGATAAG CTTGATTCAT
 601  CGAGAACGGT TTACATGTTC AATGAATCGA GTCAAATTTG TCTGCTTAAT
 651  TTTTATTGGT CACTCTTTCA CAGCCAATGA GGCACTCAAT AAACAGCGAA
 701  TAGAAATGAA ATATTTACAG TTAAAATCAA GAGTTACACT ATTGGCCGAT
 751  CTGTTTACTA AATGACTATT TAATAGTCAC AGCTCAGATA GTTAGCACTG
 801  TTTTGTCTAT TTGCAAGATG GCTGGCGAGC ATATCAAGGT GGGTTGGTGT
 851  GGTTGTTCGT CATTGTGTTG CTTGGTAAAT TTTCGAGTGT TGTTAAGGTT
 901  CCTAATTGCA GCATTTAGCG GAATAAATAT TATTCCGCTA TATGAAGGTA
 951  AATACTGAGT CCATGTTAAT TGCACATTAG ATCCGTACAT ATATTTGGCT
1001  GTGATTTTCC ACGTCATCAG GTCAGTAAGT TACGTGTTCA CACTGGGTTC
1051  TGATATAATG CACAACCATT ATAGTCACAT AGTGGTCACC GATTATGTGT
1101  TAGGTGTAGC ATTTCAGGCG ATGTTATAGT TTGTCTGTGG AATTGTTACG
1151  AGAACTTAGG GGATTCTAGA GCGTGAATCC ATCTAGTCCA TATCTCAGAG
1201  ACATGCGTCC ATGGAACAGT TATTTATTAT GTGATGTTTT GTCTGAAGTG
1251  GTTTTTTTCA AGCGTAAATT GATGTGAAAC ACAGCTGTAA TTTCTATAGG
1301  TTATCTATTT TGACGGACGC GGACGTGCTG AATCGATTCG GATGACTCTT
```

Fig. 6 (continued)

1351 GTGGCAGCTG GTGTAGACTA CGAAGATGAG AGAATTAGTT TCCAAGATTG

1401 GCCAAAAATC AAACCAACTA TTCCAGGCGG ACGATTGCCT GCAGTGAAAG

1451 TCACTGATGA TCATGGGCAC GTGAAATGGA TGTTAAGCTT GCATGCCTGC

1501 AGGTCGACTC TAGAGGATCC CCGGGTACCG GTAGAAAAAA TGAGTAAAGG

1551 AGAAGAACTT TTCACTGGAG TTGTCCCAAT TCTTGTTGAA TTAGATGGTG

1601 ATGTTAATGG GCACAAATTT TCTGTCAGTG GAGAGGGTGA AGGTGATGCA

1651 ACATACGGAA AACTTACCCT TAAATTTATT TGCACTACTG GAAAACTACC

1701 TGTTCCATGG CCAACACTTG TCACTACTTT CTCTTATGGT GTTCAATGCT

1751 TTTCAAGATA CCCAGATCAT ATGAAACGGC ATGACTTTTT CAAGAGTGCC

1801 ATGCCCGAAG GTTATGTACA GGAAAGAACT ATATTTTTCA AAGATGACGG

1851 GAACTACAAG ACACGTGCTG AAGTCAAGTT TGAAGGTGAT ACCCTTGTTA

1901 ATAGAATCGA GTTAAAAGGT ATTGATTTTA AAGAAGATGG AAACATTCTT

1951 GGACACAAAT TGGAATACAA CTATAACTCA CACAATGTAT ACATCATGGC

2001 AGACAAACAA AAGAATGGAA TCAAAGTTAA CTTCAAAATT AGACACAACA

2051 TTGAAGATGG AAGCGTTCAA CTAGCAGACC ATTATCAACA AAATACTCCA

2101 ATTGGCGATG GCCCTGTCCT TTTACCAGAC AACCATTACC TGTCCACACA

2151 ATCTGCCCTT TCGAAAGATC CCAACGAAAA GAGAGACCAC ATGGTCCTTC

2201 TTGAGTTTGT AACAGCTGCT GGGATTACAC ATGGCATGGA TGAACTATAC

2251 AAATAGCATT CGTAGAATTC TATTGCACGG TATATGGCGA AGAAACATCA

2301 TATGATGGGT GAAACAGACG AGGAATACTA TAGTGTTGAA AAGTTGATTG

2351 GTCAGGTGAG TTGAGCCTTT TATTGGTTGA TGGGATTTTA TGCTACTGGA

2401 GTCCTGTAAG TTCAGTTTGT TGCGTTGAAT AAATAAAGTT CGAATTGATA

2451 GGCGAGTGGT AACCTATGTG TTGTGATTAC TAAAAGTCAT AAGTGTGAGG

2501 TATCCCAATT ATTAGAAATG TGACTAAGAC TGAGCTTTTC TGCCTATTGG

2551 GATATTACAG ATAAGTTACT TATTTGTAGT ATCGTAATAG TGTTCACTGG

2601 GAACCATCTA ATTCACTCTC AAAAATACTT TATCGTGTCT TATTTTATTC

2651 ATCCTCATAA CTCGTTTGCT AGGAGAACAG CTGTGCCCAT AGGTTAGTTG

2701 ACAAGTTACT TACTTCCCCA TGATACGCCT TATTTTAATA ACCTAATAAA

2751 GGCTCGGTTA CGTAACGTCC CTATAGTGCA TAGCATACCT ACGAACATTG

2801 ACTCCCAACT GATTAAGTGC GGATTGCATT TTTTTGGATG TTATCGCACA

2851 GTAAGACAAT ACCATCCTCA TCTCAATGTC AAGAGGTCTT TCTCCAGGCA

Fig. 6 (continued)

2901 ACAGATCCAT ACCACCATTA CTTACATCCA TCAGAGCTGT TCCCAGAATG

2951 TCTTGTTATA AACGGATGGA TTATTTATTT ATTTGACCAC ATAAATATTG

3001 CATCAAAGAG GTGGGGGATC CACTAGTTCT AGAGCGGCCG CCACCGCGGT

3051 GGAGGATCTG AATCCGACCA ACCGTTCTAT GAAAATCGTT GTATCTCCGA

3101 AACCACTGGA CGGATTTTTA TGATGTTTGT TTTAGATTAT TTGCGAGAGC

3151 GTGGGCGTTA ATATAAAACA AGAATGATCT GAATCCGACC AACCGTTCTA

3201 TGAAAATCGT TGTATCTCCG AAACCACTGG ACGGATTTTT ATGATGTTTG

3251 TTTTAGATTA TTTGCGAGAG CGTGGGCGTT AATATAAAAC AAGAATGATC

3301 TGAATCCGAC CAACCGTTCT ATGAAAATCG TTGTATCTCC GAAACCACTG

3351 GACGGATTTT TATGATGTTT GTTTTAGATT ATTTGCGAGA GCGTGGGCGT

3401 TAATATAAAA CAAGAATCAT CTCAATCCCA TCAGCCGTTC TATGAAAATC

3451 GTTGTATCTC CGAAACCACT GGACGGATTT TTATGATGTT TGTTTTAGAT

3501 TATTTGCGAG AGCGTGGGCG TTAATATAAA ACAAGAATGA TCTGAACACG

3551 GGTGTTTTTC TGTTCAGCTT ATGCAACTTT AAAATTCGAT GGGTCGTCTC

3601 AACGAAATTT GTATTGCTTT GCTCCAGCTT TTGTTCCCTT TAGTGAGGGT

3651 TAATTGCGCG CTTGGCGTAA TCATGGTCAT AGCTGTTTCC TGTGTGAAAT

3701 TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA GCATAAAGTG

3751 TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC

3801 GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA

3851 TGAATCGGCC AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCTCTTC

3901 CGCTTCCTCG CTCACTGACT CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG

3951 CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG

4001 GATAACGCAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA

4051 CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG

4101 ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCGACAG

4151 GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT

4201 CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC

4251 GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTCGG

4301 TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGACCC CCCGTTCAGC

4351 CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA

4401 AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG

4451 AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT

Fig. 6 (continued)

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4501  ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA
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4601  CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA
4651  AAAAAGGATC TCAAGAAGAT CCTTTGATCT TTTCTACGGG GTCTGACGCT
4701  CAGTGGAACG AAAACTCACG TTAAGGGATT TTGGTCATGA GATTATCAAA
4751  AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA
4801  TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC
4851  AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT CCATAGTTGC
4901  CTGACTCCCC GTCGTGTAGA TACTACGATA CGGGAGGGCT TACCATCTGG
4951  CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT
5001  TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT
5051  GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG
5101  AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA
5151  CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC
5201  GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA
5251  AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG
5301  CAGTGTTATC ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC
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5801  ACATTTCCCC GAAAAGTGCC ACCTGACGCG CCCTGTAGCG GCGCATTAAG
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5901  CCCTAGCGCC CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC
5951  GCCGGCTTTC CCCGTCAAGC TCTAAATCGG GGGCTCCCTT TAGGGTTCCG
6001  ATTTAGTGCT TTACGGCACC TCGACCCCAA AAAACTTGAT TAGGGTGATG
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Fig. 6 (continued)

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6101  TTGGAGTCCA CGTTCTTTAA TAGTGGACTC TTGTTCCAAA CTGGAACAAC
6151  ACTCAACCCT ATCTCGGTCT ATTCTTTTGA TTTATAAGGG ATTTTGCCGA
6201  TTTCGGCCTA TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG
6251  AATTTTAACA AAATATTAAC GCTTACAATT TCCATTCGCC ATTCAGGCTG
6301  CGCAACTGTT GGGAAGGGCG ATCGGTGCGG GCCTCTTCGC TATTACGCCA
6351  GCTGGCGAAA GGGGGATGTG CTGCAAGGCG ATTAAGTTGG GTAACGCCAG
6401  GGTTTTCCCA GTCACGACGT TGTAAAACGA CGGCCAGTGA GCGCGCGTAA
6451  TACGACTCAC TATAGGGCGA ATTGGGTACC GGGCCCCCCC TCGA
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Fig. 7

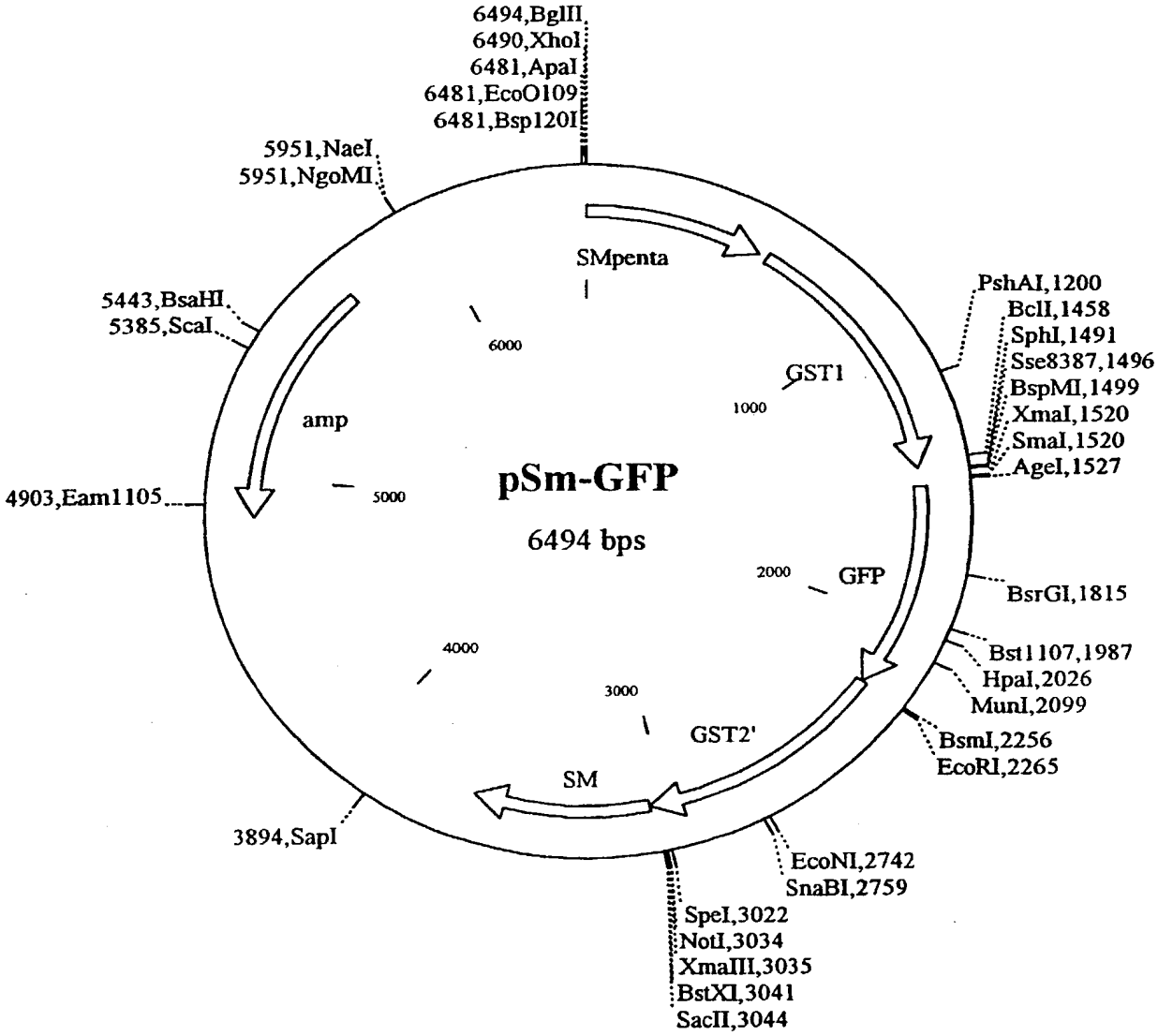
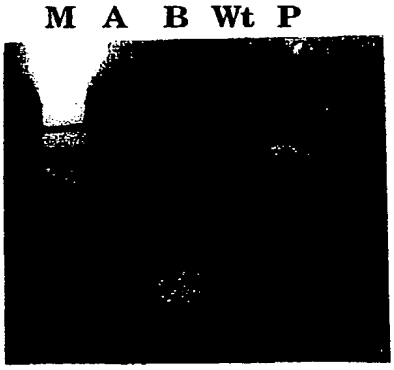

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HpaI,2026
MunI,2099
BsmI,2256
EcoRI,2265
EcoNI,2742
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Fig. 8


M A B Wt P

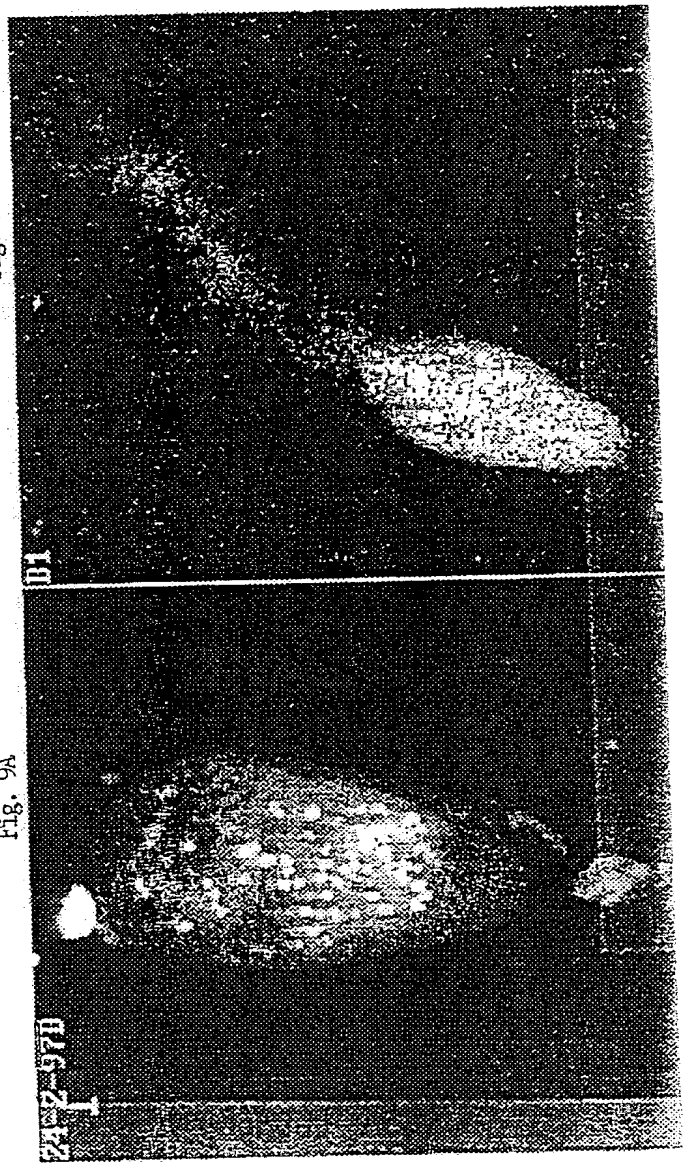


Fig. 9B

Fig. 9A



Fig. 9D

Fig. 9C

Fig. 10B

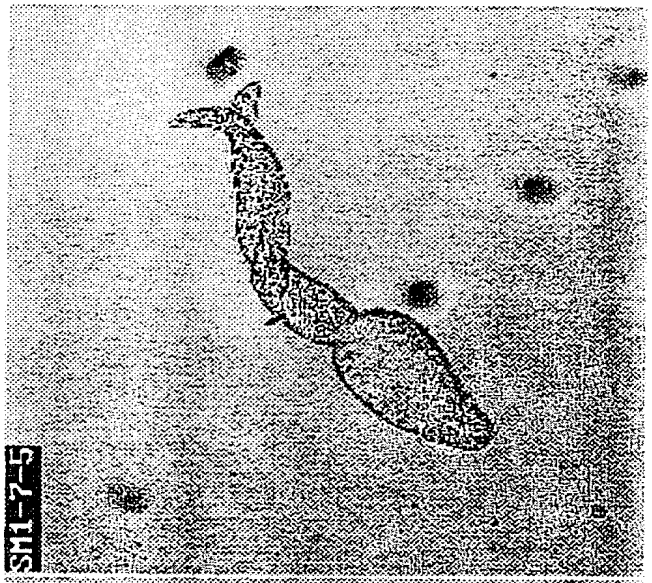
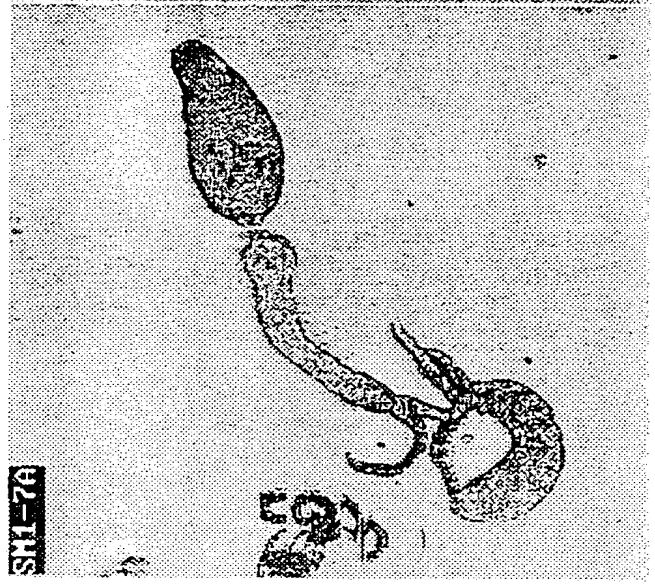


Fig. 10A



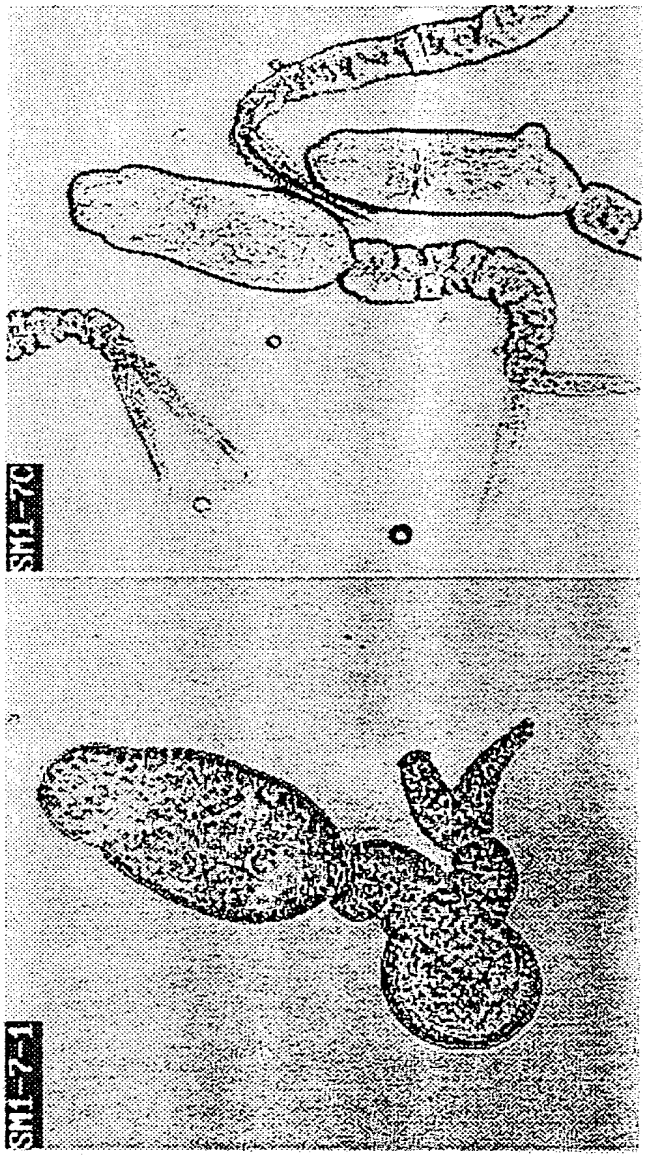


Fig. 10D

Fig. 10C

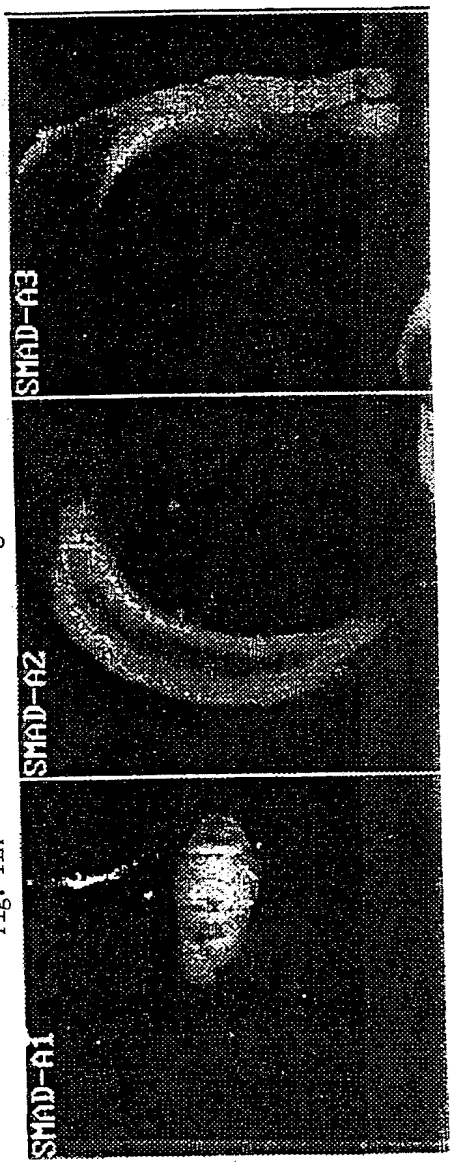


Fig. 11A

Fig. 11B

Fig. 11C

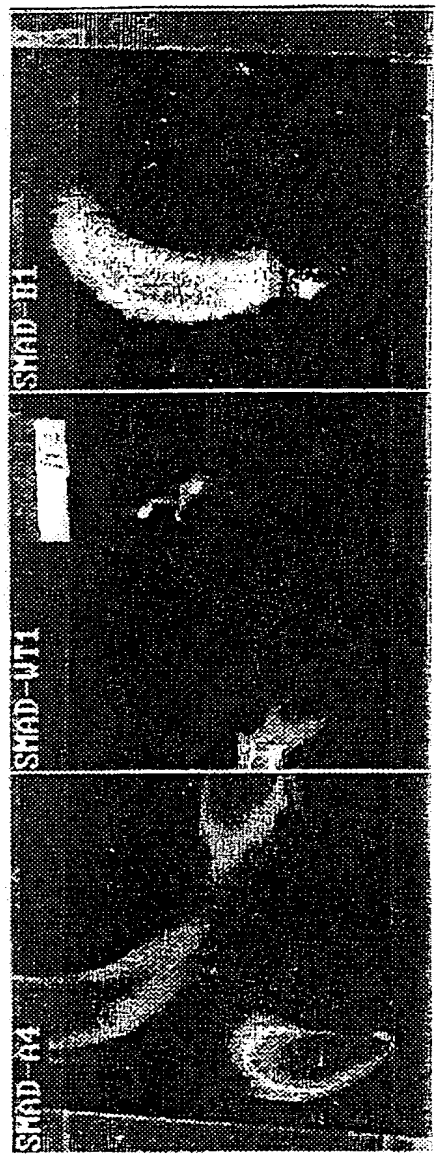


Fig. 11D Fig. 11E Fig. 11F

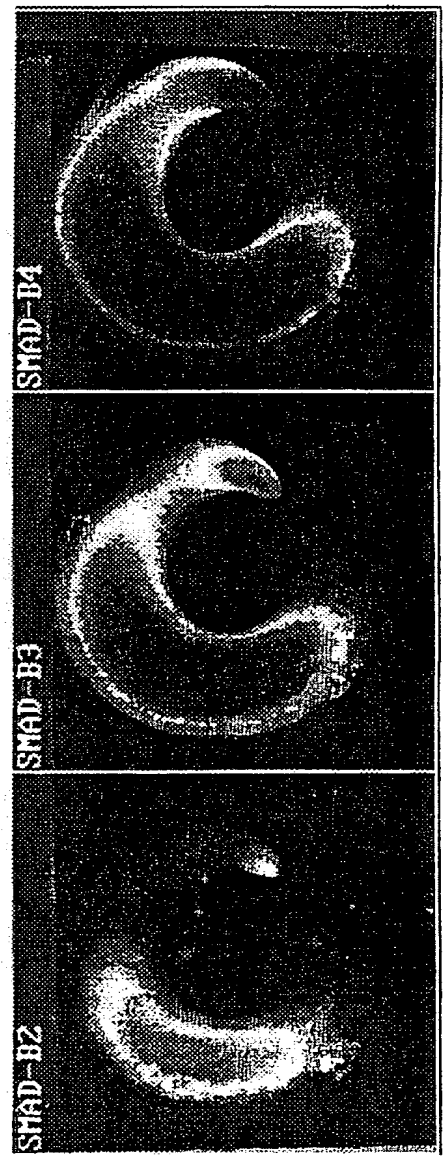


Fig. 11G Fig. 11H Fig. 11I

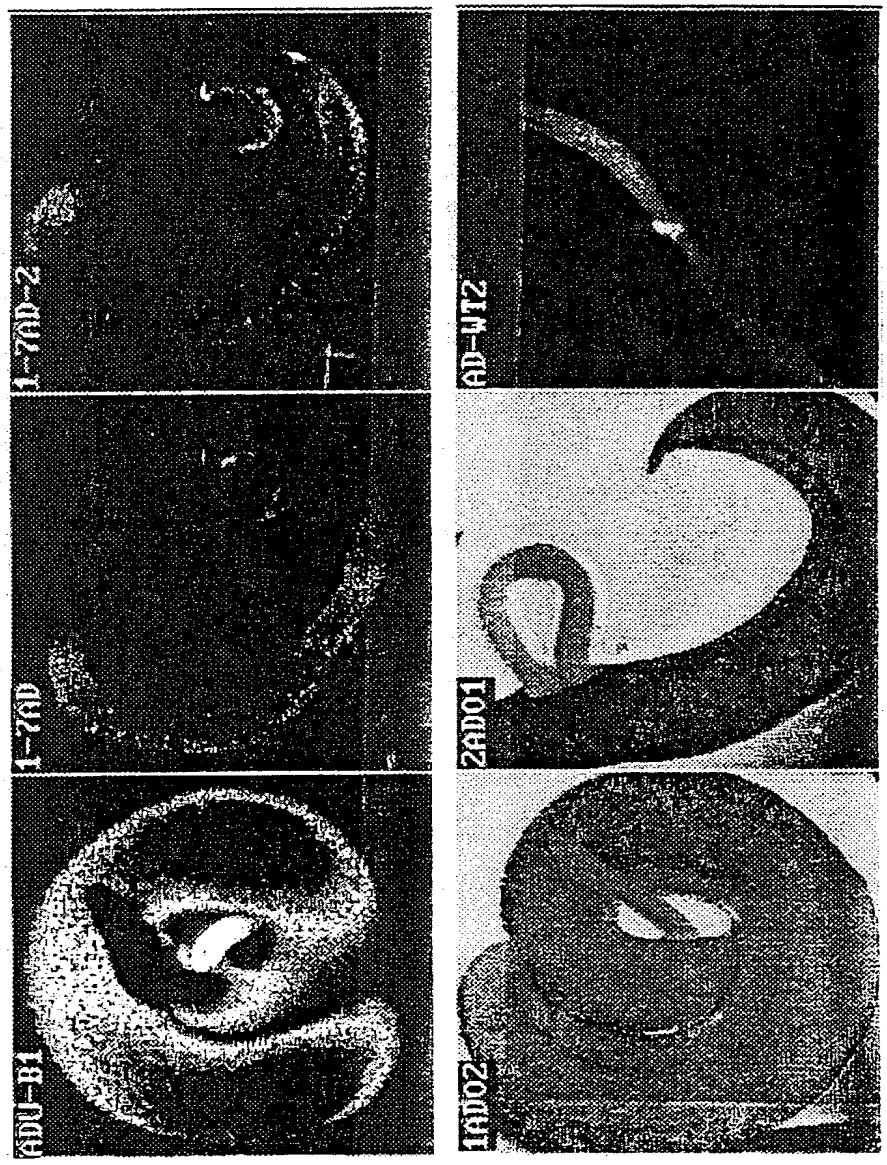


Fig. 12A Fig. 12B Fig. 12C

Fig. 12D Fig. 12E Fig. 12F

Fig. 13A



Fig. 13B



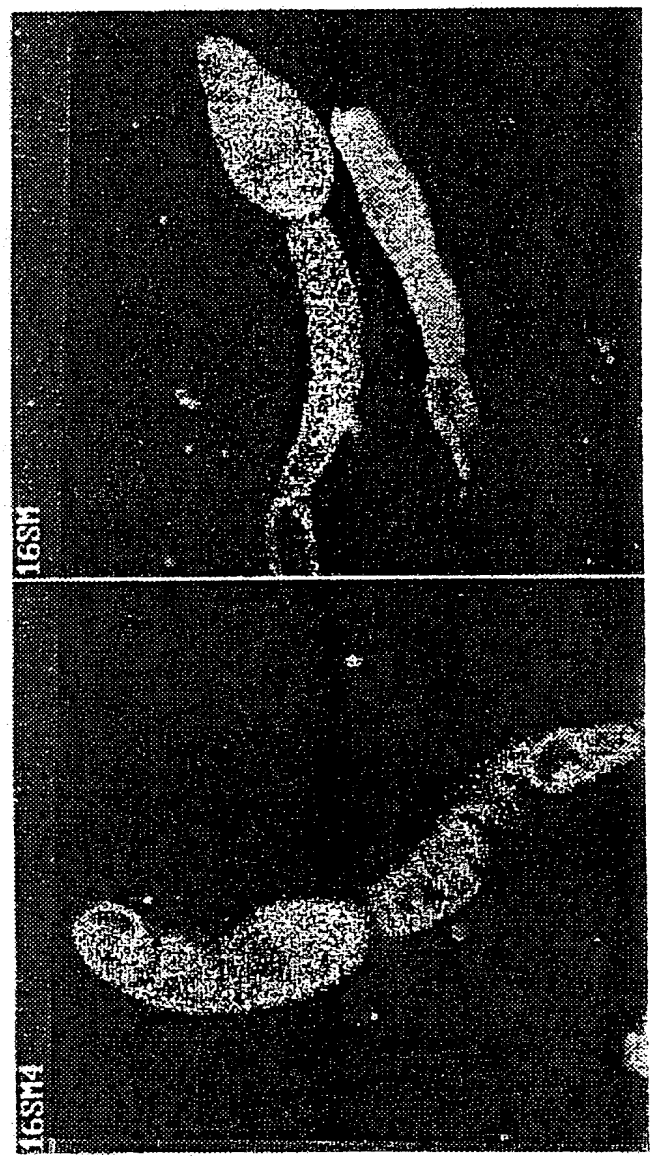


Fig. 13D

Fig. 13C

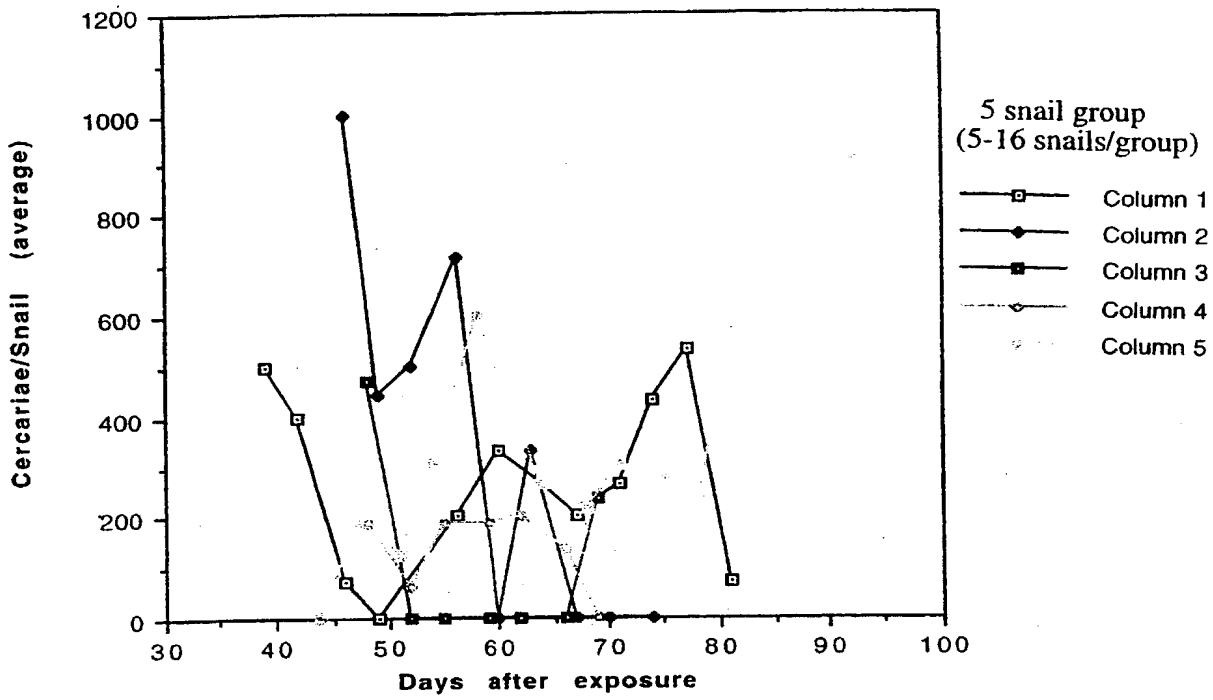


Fig. 14

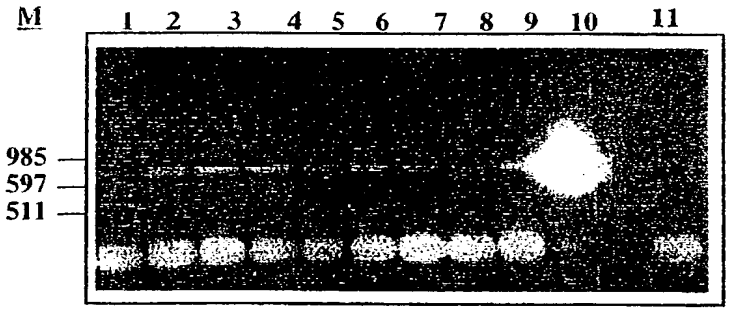


Fig. 15

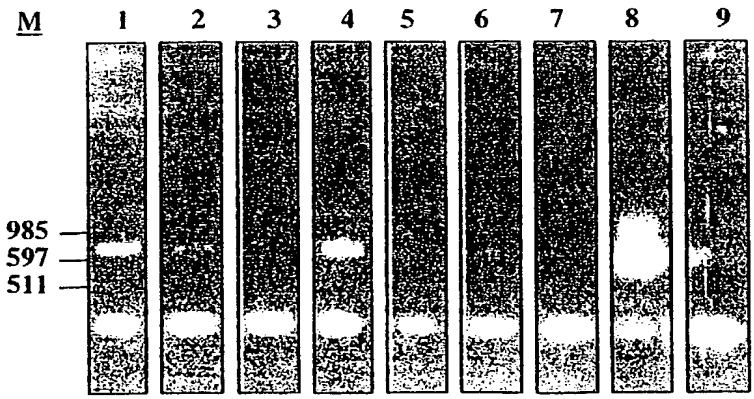


Fig. 16

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Joseph Hamburger and Avraham Laban

METHODS FOR BULK STABLE INTRODUCTION AND EXPRESSION OF FOREIGN GENES INTO EUKARYOTIC PARASITES FOR *IN VIVO* PRODUCTION OF DESIRED GENE PRODUCTS

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Mark M. Friedman c/o Anthony Castorina

(B) STREET: 2001 Jefferson Davis Highway, Suite 207

(C) CITY: Arlington

(D) STATE: Virginia

(E) COUNTRY: United States of America

(F) ZIP: 22202

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 1.44 megabyte, 3.5" microdisk

(B) COMPUTER: Twinhead® Slimnote-890TX

(C) OPERATING SYSTEM: MS DOS version 6.2, Windows version 3.11

(D) SOFTWARE: Word for Windows version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 09/201,850

(B) FILING DATE: December 1, 1998

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Friedmam, Mark M.

(B) REGISTRATION NUMBER: 33,883

(C) REFERENCE/DOCKET NUMBER: 325/71

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 972-3-5625553

(B) TELEFAX: 972-3-5625554

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5356

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6494

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA  4400
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AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT  4500
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CAGTGGAACG AAAACTCACG TTAAGGGATT TTGGTCATGA GATTATCAAA  4750
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GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG  5100
AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA  5150
CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC  5200
GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA  5250
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ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC  5400
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GGTTTTCCCA GTCACGACGT TGTAAAACGA CGGCCAGTGA GCGCGCGTAA  6450
TACGACTCAC TATAGGGCGA ATTGGGTACC GGGCCCCCCC TCGA        6494
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATCGAGAAC GGTTTACATG TTCA 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCAGCCTCCT CACATGCTCC A 21

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
ATGAGTAAAG GAGAAGAACT TTTC 24

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
TTTGTATAGT TCATCCATGC C 21

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
TCTCCCATGA TGTATACATT ATGT 24

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
TCTCCATCGA AGGGTCATCA CG 22

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
GATCTCACCT ATCAGACGAA ACAAAGAAAA TTTTAAAATT GTTGGTGGAA 50
GTGCCTGTTT CGCAATATCT CCGGAATGGT TGGTCGTATC GTTGTGAAAA 100
TTGTTTCATA TTATTGGTGA C 121

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00651

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :800/4, 13, 21, 24, 25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/4, 13, 21, 24, 25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, CAPLUS, BIOSIS, MEDLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/11191 A1 (MILLER I.) 27 March 1997, entire document.	1-36
Y		37-52
Y	MUDGETT et al. Electroporation of Embryonic Stem Cells for Generating Transgenic Mice and Studying In Vitro Differentiation, Methods in Molecular Biology, November 1995, Vol 48, pages 167-184, entire document.	37-52
A	KAPPEL et al. Regulating Gene Expression in Transgenic Animals, Current Opinion in Biotechnology, 1992, Vol 3, pages 548-553.	1-52
A	PURSEL et al. Expression and Performance in Transgenic Pigs, J. Reprod. Fert., 1990, Vol Suppl, 40, pages, 235-245, entire document.	1-52

[X] Further documents are listed in the continuation of Box C. [] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 FEBRUARY 2000	Date of mailing of the international search report 04 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHIN-LIN CHEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00651

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PALMITER et al. Metallothionein-Human GH Fusion Genes Stimulate Growth of Mice, Science, 18 November 1983, Vol 222, pages 809-814, entire document.	1-52

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL99/00651

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/00; A01K 67/00; C12N 15/00